

Intratumoral T cell activation kills tumors regardless of T cell specificity

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Immunotherapies putatively require tumor-specific T cells. Here we show how T cells can eliminate tumors without tumor specificity via paracrine signaling. Activating unexhausted bystander non-tumor-specific T cells within tumors resulted in tumor elimination without conventional recognition-dependent mechanisms and in the absence of any tumor-specific T cell receptor (TCR) $\alpha\beta^+$ T cells. Robust T cell activation recruited immune cells, used innate leukocytes and triggered a tumoricidal combination of effector molecules and panoptotic pathways. Mechanistically, interferon- γ , tumor necrosis factor and nitric oxide induced caspase-dependent death, recapitulating melanoma clearance in mice or human melanoma cell death in vitro. Gene expression signatures associated with this response in mice were predictive of survival among human patients with melanoma. Thus, triggering productive T cell activation within tumors can be sufficient for immunotherapy, without needing to induce or rescue cancer-specific responses.

T cell-mediated killing of tumors is promoted therapeutically through adoptive T cell transfers, tumor antigen vaccines and immune checkpoint blockade (ICB). These and other immunotherapies have shown remarkable success for some patients. However, each relies on the presence of sufficiently immunogenic tumor antigens and tumor-reactive T cells. Efforts to leverage the innate arm of the immune system using agonists of innate sensing pathways (for example, cGAMP and CpG) are believed to depend on tumor-specific CD8⁺ T cells^{1–4}, and

have demonstrated limited clinical efficacy thus far. Unfortunately, tumor-specific T cells may not be present, sufficiently abundant or functional. Promising efforts to prime or boost tumor-specific responses through vaccination are in development^{5,6} but require the personalized identification of immunogenic tumor antigens and a patient with a healthy immune system.

Humans accumulate memory T cells throughout their lives as they experience microbial infections. These T cells survey most of the body,

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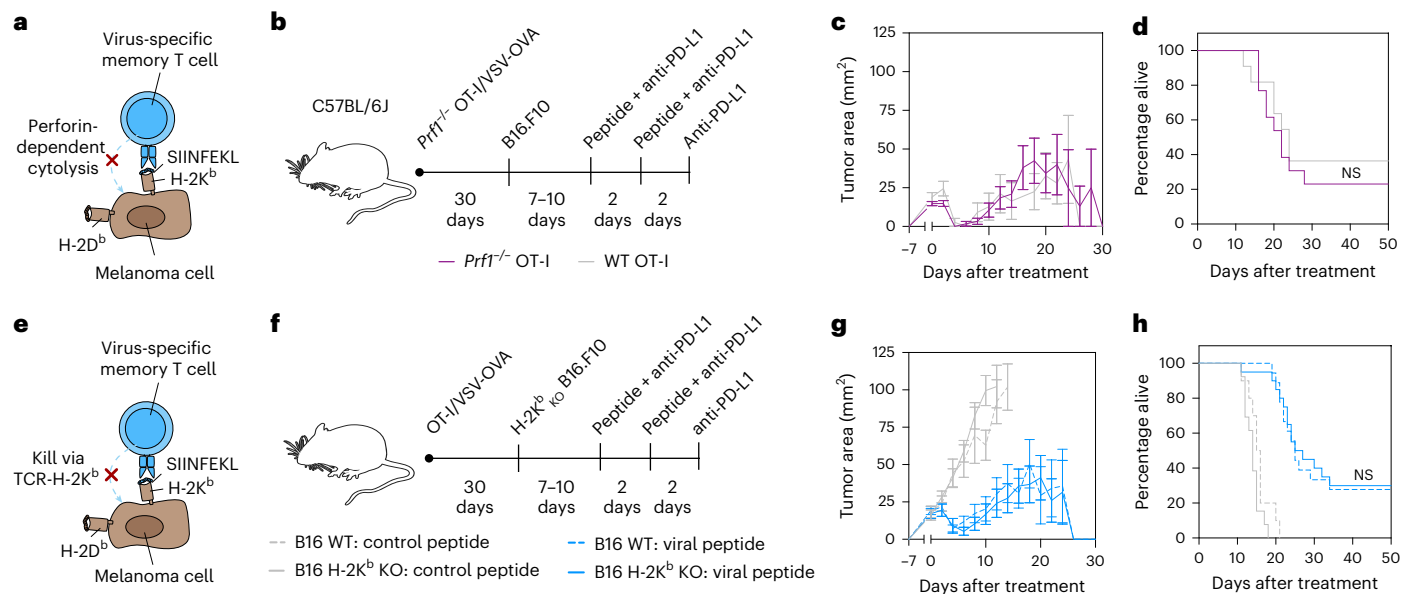


Fig. 1 | Activated virus-specific CD8⁺ T cells eradicate tumors without tumor recognition. **a,e**, Hypothesis schematics: perforin-dependent (**a**) and TCR:H-2K^b-dependent (**e**) killing. **b,f**, Perforin (**b**) and H-2K^b (**f**) experimental schematics. **c,d**, Tumor growth (**c**) and survival (**d**) of B16.F10 tumor-bearing *Prfl*^{-/-} (purple, *n* = 14) or WT (gray, *n* = 10) OT-I memory mice. **g,h**, Tumor growth (**g**) and survival (**h**) of B16.F10 tumor-bearing OT-I memory mice given anti-PD-L1 with viral

peptide (blue) or irrelevant peptide (gray), bearing H-2K^b KO (solid, *n* = 20 and 13, respectively) or WT guide RNA (gRNA) (dashed, *n* = 18 and 10) tumors. All data are from two independent experiments. Tumor growth data are shown as the mean ± s.e.m. **d,h**, *P* values were determined using a log-rank (Mantel–Cox) test; NS, *P* > 0.05. NS, not significant.

looking for evidence of reinfection. When pathogen-specific memory T cells re-encounter cognate antigen, they trigger potent local immune responses^{7–10}. Several recent studies reported that virus-specific T cells extend their surveillance to transformed tissue, including memory T cells that were naturally established because of previous exposures to influenza A virus (IAV), Epstein–Barr virus (EBV) and cytomegalovirus (CMV)^{11–13}. Almost all adults have memory T cells that recognize at least one of these pathogens^{11,14}. Unlike tumor antigens, which are often unique to individual patients, these common viral infections exhibit more consistent, well-annotated antigens among individuals of a similar haplotype.

When virus-specific memory T cells are reactivated with unadjuvanted cognate peptides (representing a few epitopes from IAV, EBV or CMV), a robust inflammatory response is elicited in human tumor explants¹¹. Intratumoral delivery of non-adjuvanted viral peptide into mouse tumors can reactivate intratumoral ovalbumin (OVA)-specific or endogenous virus-specific memory CD8⁺ T cells, and eliminate orthotopic and autochthonous tumors. When used in combination with programmed death-ligand 1 (PD-L1) blockade, viral peptide injection cured otherwise ICB-resistant tumors¹¹. These findings have been recapitulated using several alternative models, with mechanisms being putatively attributed to direct tumor cell killing by redirected virus-specific T cells or epitope spreading and amplification of tumor-specific T cell populations^{15–19}.

In this study, we asked how non-tumor-specific T cells trigger killing and elimination of tumors. Contrary to expectation, neither perforin-dependent killing by virus-specific T cells nor peptide presentation by cancer cells was required. Rather, we describe a distinct paracrine mechanism according to which T cells kill tumor cells, achieving tumor elimination in the absence of any classical major histocompatibility (MHC)-dependent tumor cell recognition, relying instead on multiple innate mechanisms mediated by a combination of effector molecules to induce apoptosis. We propose that tumor cell antigen specificity might not always be required for T cell-driven immunotherapies, provided that robust T cell activation occurs within the tumor microenvironment (TME).

Results

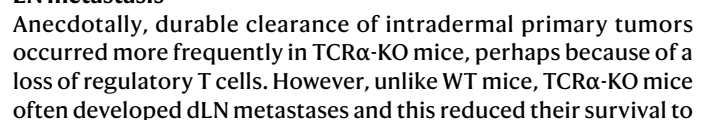
Virus-specific CD8⁺ T cells clear tumors without tumor recognition

When tumor-infiltrating, virus-specific memory CD8⁺ T cells are activated with peptide in the presence of anti-PD-L1 therapy, tumors that lack expression of cognate viral antigen are killed¹¹. We wanted to understand the mechanism(s) by which the activation of non-tumor-specific memory T cells causes the destruction of tumors (Fig. 1a). We first reasoned that memory T cells may kill tumors through T cell receptor (TCR):MHC-dependent cytotoxicity (which canonically occurs through perforin-dependent processes) after injected viral peptides displace H-2K^b-bound peptides. To test this, we established trackable populations of virus-specific memory T cells by transferring either naive wild-type (WT) OT-I T cells or perforin-deficient OT-I T cells, which both recognize the OVA-derived SIINFEKL peptide presented by H-2K^b, into C57BL/6J recipient mice (Fig. 1b). We then infected those recipients with vesicular stomatitis virus expressing OVA (VSV-OVA); 30 days later, we intradermally implanted B16 cells, which do not express OVA. When perforin-knockout (KO) OT-I memory mice were treated with viral peptide in combination with anti-PD-L1, mice were as capable of promoting durable tumor clearance as WT OT-I immune chimeras (Fig. 1c,d and Extended Data Fig. 1a).

Because alternative mechanisms of CD8⁺ T cell-mediated cytotoxicity exist, we took a comprehensive approach to test whether viral peptide recognition on tumor cells by virus-specific OT-I T cells is even required for elimination (Fig. 1e). To do so, we knocked out the MHC class I allele capable of presenting SIINFEKL, H-2K^b, in B16 melanoma cells using CRISPR–Cas9 (Fig. 1f and Extended Data Fig. 2). In the absence of H-2K^b, tumor cells cannot present MHC-bound SIINFEKL. WT and H-2K^b KO B16 tumors were equally susceptible to clearance after peptide injection (Fig. 1g,h and Extended Data Fig. 1b). Thus, tumor clearance occurs in the absence of direct OT-I TCR-dependent recognition of the tumor cells.

Tumors are eliminated without classical T cell recognition

We next considered whether tumor elimination depended on the induction of endogenous tumor-specific T cells. In support of this



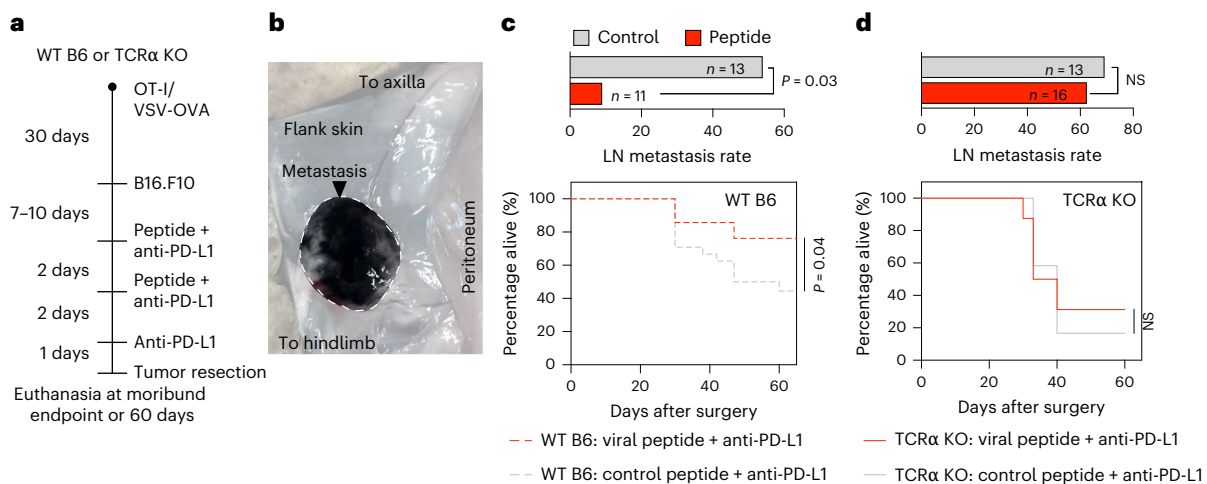


Fig. 3 | Endogenous T cells are required for protection against LN metastasis. **a**, Tumor resection experimental schematic. After primary B16 tumor resection, mice were euthanized at moribund endpoints or 60 days after surgery and dissected to assess metastases. **b**, Representative image of flank tdLN metastasis at euthanasia. **c,d**, LN metastasis rate (top) and survival (bottom) in WT B6 mice

(**c**; two experiments, $n = 11$ and 13) or TCRα-KO mice (**d**; one experiment, $n = 16$ and 13) treated with viral (red) or control (gray) peptide before tumor resection. P values were determined using a two-sided Fisher's exact (metastasis rate) or log-rank (Mantel–Cox) (survival) test; NS, $P > 0.05$.

the rate of WT mice. This led us to test whether viral peptide treatment with anti-PD-L1 therapy prevented mortality from metastasis and whether this process depended on endogenous T cells. When we surgically resected B16 melanoma, 54% of mice developed metastases in the tumor-draining LN (tdLN) at the time of moribund euthanasia (Fig. 3a–c). Viral peptide treatment with anti-PD-L1 before tumor resection reduced the rate of LN metastases in WT B6 mice (Fig. 3c), but not in TCRα-KO mice (Fig. 3d). Thus, while endogenous T cells are not required for viral peptide treatment to clear primary tumors, they are required for locoregional protection of LNs and prevention of metastatic outgrowth after intratumoral viral peptide treatment.

Virus-specific CD8⁺ T cells kill tumor cells via cytokines and nitric oxide

To systematically profile the TME response to virus-specific CD8⁺ tumor-infiltrating lymphocyte (TIL) activation, we performed single-cell CITE sequencing (CITE-seq) analysis of sorted tumor cells and leukocytes from tumors of mice treated with anti-PD-L1 and intratumoral injection of (1) viral peptide that reactivates intratumoral virus-specific memory T cells, (2) control peptide as a negative control or (3) a Toll-like receptor 9-agonist (CpG) (Fig. 4a, Extended Data Fig. 4 and Supplementary Table 1). Virus-specific CD8⁺ TILs activated in response to intratumoral viral peptide injection, detected using both flow cytometry and CITE-seq transcriptomics (Extended Data Fig. 5a,b). Unsupervised clustering and abundance analysis of melanoma cells identified a subpopulation, melanoma 5, enriched in tumors after viral peptide injection (Fig. 4b,c). Gene set enrichment and subsequent expression analyses identified multiple major cell death pathways and genes specific to viral peptide treatment (Fig. 4d,e). Treatment-induced melanoma 5 cells exhibited increased expression of driver genes of pyroptosis (*Gsdmd*)²⁰, apoptosis (*Tnfrsf1a*, *Zbp1*, *Casp7*, *Casp8*)²¹ and necroptosis (*Ripk1*, *Mkl1*)^{22–24} (Fig. 4e). Known regulators of the oxidative stress response (*Mt2*, *Nfe2l1*, *Gpx1*) were also increased²⁵.

Cell death pathways were associated with tumor necrosis factor (TNF) and interferon-γ (IFNγ) responses, and dual (but not individual) neutralization of TNF and IFNγ prevented tumor clearance (Fig. 4f–h and Extended Data Fig. 6a–d). Given that treatment-induced melanoma cells also upregulated oxidative stress pathways, and that nitric oxide (NO) can synergize with cytokines to induce cell death^{26–29}, we assessed the role of NO by inhibiting inducible NO synthase (iNOS) with

L-NIL (Fig. 4i,j). iNOS inhibition reduced tumor debulking, although the trend toward decreased survival was non-significant (Fig. 4j). Of note, CpG with anti-PD-L1 treatment did not eliminate B16 melanoma tumors¹¹, and we observed that it did not induce TNF and IFNγ synergistic responses (Extended Data Fig. 7a–c).

We next tested whether soluble factors produced by T cells were sufficient to induce tumor cell death (Fig. 4k). We stimulated virus-specific memory T cells with peptide ex vivo; after 30 h, we collected the supernatant and then applied it to B16 tumor cells in vitro. The supernatant from activated virus-specific memory T cells alone increased tumor cell death from 16% to 40% as measured using annexin V staining, which indicates apoptosis (Fig. 4k). Adding the NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) further increased death to 75%. We next tested whether killing depended on the activity of TNF and IFNγ. Blocking TNF and IFNγ partially reduced killing. Thus, TNF and IFNγ partly explain T cell autonomous killing and exogenous NO further increases cell death.

To assess whether cytokines and NO alone could directly induce tumor cell death, we performed the reciprocal experiment by treating tumor cells in vitro with recombinant cytokines and SNAP (Fig. 4l). Treatment with TNF, IFNγ or SNAP alone had no effect, but co-treatment with TNF and IFNγ led to 64% annexin V⁺ tumor cells. The addition of exogenous NO using SNAP increased the fraction of cells that stained double-positive for annexin V and propidium iodide (PI) after TNF and IFNγ co-treatment, indicating that NO increases late-stage apoptosis or necrosis. Similar results were seen with YUMM1.7 melanoma cells (Extended Data Fig. 8a). While cytokines may have induced production of NO by tumor cells, blocking NO production with L-NIL did not affect TNF–IFNγ-mediated killing of B16 cells in vitro (Extended Data Fig. 8b). Because L-NIL treatment inhibits tumor debulking in vivo (Fig. 4j), it is likely that non-tumor and non-antiviral T cells are a source of NO in vivo. We next tested which cell types in the TME produce cytokines and NO in response to viral peptide. At the protein level, OT-I cells produced IFNγ and TNF, whereas myeloid cells were the major source of NO (Extended Data Fig. 8c–e). Transcriptional analysis also indicated that myeloid cells were a contributor of TNF (Extended Data Fig. 8f).

Based on these findings, we tested whether the combination of soluble mediators could recapitulate tumor death in vivo. Intratumoral delivery of TNF, IFNγ and SNAP into B16 tumors in naive mice mimicked the tumor control kinetics observed in response to viral peptide and was sufficient to eliminate tumors in 29% of mice (Fig. 4m).

This prompted us to test whether cancer cells have a cell-intrinsic sensitivity to this form of cell death compared to healthy cells. We compared cytokine responses in the human melanoma cell line SK-MEL-2 and primary human melanocytes (Fig. 4n). In healthy melanocytes, co-treatment with TNF, IFN γ and SNAP increased the percentage of annexin V⁺/PI⁺ cells from 4.9% to 12.5% (2.6-fold). In contrast, treatment increased annexin V⁺/PI⁺ melanoma cells from 5.7% to 38.6% (6.8-fold). These results suggest that human melanoma cells are intrinsically more sensitive to effector cytokines in vitro than healthy melanocytes. Similar results were seen with A375 human melanoma cells (Extended Data Fig. 8g).

To define the pathways according to which tumor cells are dying, we treated B16 cells in vitro with a panel of inhibitors after IFN γ + TNF + SNAP exposure (Fig. 4o). RIPK3 (GSK'872) and ferroptosis (Lip-1) inhibitors had no effect, while the pan-caspase inhibitor zVAD eliminated nearly all cell death, indicating a caspase-dependent mechanism. This mechanism was conserved in human cells, as IFN γ + TNF + SNAP triggered a caspase-dependent death in the human melanoma cell line SK-MEL-2 (Extended Data Fig. 8h). Taken together, these data suggest that tumor eradication by virus-specific T cells uses the combined activity of IFN γ , TNF and NO, which induce apoptosis in mouse and human melanoma cells, and are sufficient to eliminate B16 tumors in vivo.

VCAM-1 and myeloid cells are essential

We wished to further understand whether local memory T cells autonomously kill tumor cells or whether the activation of additional effector cell types was essential. We first used CITE-seq to assess changes in TME cell types after treating tumors with peptide and anti-PD-L1 in vivo. We observed a proportional increase in activated virus-specific memory T cells (Extended Data Fig. 9a), and myeloid cell populations increased pronouncedly. When we examined the specific subsets driving this shift, we identified *Ly6c2*-expressing and *Nos2*-expressing monocytes as the major infiltrators (Fig. 5a).

We next examined the ability of activated virus-specific memory T cells to directly signal to these effector myeloid populations based on ligand–receptor expression (Fig. 5b). This analysis provided a potential mechanism according to which activated OT-I cells can recruit (for example, via chemokines) and signal to (for example, via IFN γ) innate mediators. This T cell–myeloid interaction was unique to activated virus-specific T cells (Extended Data Fig. 9b,c). In support of cell–cell interactions underlying therapy response, peptide provoked accumulation of neutrophils and myeloid cells (Extended Data Fig. 9d), which appeared to colocalize with reactivated CD45.1⁺ virus-specific T cells in the TME (Fig. 5c). Depletion of inflammatory monocytes and neutrophils using the combination of clodronate liposomes and anti-Ly6G prevented tumor clearance (Fig. 5d and Extended Data Fig. 9e,f), demonstrating that contributions of innate immune cells are essential. Local memory CD8⁺ T cell populations are known to recruit leukocytes

to tissues through IFN γ -induced VCAM-1 upregulation by endothelial cells⁷; myeloid cells use VCAM-1 to enter tissue.³⁰ Consistent with the hypothesis that recruitment of cells to the TME contributed to therapeutic effects in vivo, VCAM-1 neutralization limited tumor clearance in OT-I memory mice (Fig. 5e). Determining which specific leukocyte populations are recruited in a VCAM-1-dependent manner will require further investigation.

We previously showed that anti-PD-L1 therapy is required during viral peptide treatment to achieve complete tumor elimination.¹¹ To further understand the mechanism of this requirement, we used CITE-seq to examine changes in the TME cell types after treating tumors with peptide $\pm$ anti-PD-L1. PD-L1 was expressed broadly across host and tumor cells (Extended Data Fig. 10a). Among all cell types, *Ly6c2*-expressing monocytes and melanoma 5 cells exhibited the largest gene expression changes with the addition of anti-PD-L1 therapy to viral peptide treatment at 12 h (Extended Data Fig. 10b,c). Anti-PD-L1 increased ISG and *Tnf* expression on *Ly6c2*-expressing monocytes, chemokine expression on activated OT-Is and decreased pro-survival *Bcl2* expression on melanoma cells (Extended Data Fig. 10d), providing multiple potential mechanisms according to which PD-1/PD-L1 pathways contribute to a paracrine killing mechanism (for example, via increasing the amount of tumoricidal signals, such as *Tnf* produced by monocytes).

Tumoricidal signatures in mice are predictive of survival in patients with melanoma

We wondered whether similar immunological mechanisms to those induced by activated virus-specific T cells in our mouse model may occur in human tumors. We queried the gene expression signatures of our driver cell types in the Cancer Genome Atlas (TCGA) human skin melanoma dataset. Stratification according to signature scores for activated virus-specific T cells, *Ly6c2* monocytes, effector neutrophils and melanoma cluster 5 (Supplementary Table 2), as well as a combination of all signatures, predicted overall patient survival (Fig. 6a,b). To exclude the artifact that this was simply due to general immune cell infiltration, we also defined control signatures for each cell type at baseline in our control-treated samples (Methods). These showed no predictive power. Lastly, to determine whether these effector cell states co-occur in human tumors, we calculated the correlation of individual cell type signatures across skin melanoma tumors. We observed a strong positive correlation, supporting that the cascade of tumoricidal paracrine signaling we described in this study occurs in human tumors with favorable prognoses (Fig. 6c).

Discussion

We show that robust T cell activation within tumors induced tumor clearance, regardless of whether any T cells recognize antigen displayed by tumor cells, through a paracrine mechanism involving effector cytokines, NO, leukocyte recruitment and innate immune cells. It must

Fig. 4 | Virus-specific CD8⁺ T cells kill tumor cells via cytokines and nitric oxide.

a, CITE-seq experimental schematic. **b**, Uniform manifold approximation and projection (UMAP) embedding of B16 melanoma clusters ($n = 20,631$ cells). **c**, Proportions of B16 melanoma clusters across experimental conditions (one CITE-seq experiment; $n = 2–4$ mice per group). **d,e**, Expression of programmed cell death pathways (**d**) and selected genes (**e**) across melanoma clusters. **f**, Scaled cytokine response signature score, comparing cluster melanoma 5 with each other cluster; $P < 2.22 \times 10^{-16}$ for all comparisons except TNF (melanoma 5 versus melanoma 6, $P = 3.4 \times 10^{-3}$) and oxidative stress (melanoma 5 versus melanoma 6, $P = 2.6 \times 10^{-13}$). **g,i**, Treatment schematics for cytokine-neutralizing antibodies (**g**) and L-NIL (**i**). **h,j**, Tumor growth (left) and survival (right) of mice treated with anti-TNF (XT3.11) plus anti-IFN γ (XMGL2) (blue, $n = 14$) or isotype control (red, $n = 15$) (**h**), or with L-NIL (blue, $n = 25$) or PBS (red, $n = 23$) (**j**), during viral peptide + anti-PD-L1 therapy; two experiments each. **k**, Reactivated T cell supernatant assay: schematic (top) and annexin V⁺ B16 cells

30 h after the indicated treatments (bottom). Supp, supernatant. **l**, In vitro cytokine treatment: schematic (top), annexin V⁺ (middle) and annexin V⁺/PI⁺ (bottom) B16 cells after the indicated treatments. **m**, Tumor growth (left) and survival (right) of mice given two intratumoral injections of recombinant IFN γ (1 μ g), TNF (0.5 μ g) and SNAP (1 μ g) (red, $n = 23$, three experiments) or PBS (gray, $n = 5$, two experiments). **n**, In vitro cytokine treatment: schematic (left) and annexin V⁺/PI⁺ frequencies in SK-MEL-2 melanoma cells (red) or primary human melanocytes (blue) after PBS or IFN γ + TNF + SNAP (right). **o**, In vitro death pathway inhibition: schematic (left) and annexin V⁺ B16 cells treated with IFN γ + TNF + SNAP plus the indicated inhibitors (right). **k,l,i,n,o**, Data are from technical replicates within one experiment ($n = 3$; $n = 2–3$ in **l**). **c,n,o**, Data are the mean $\pm$ s.e.m. **k,l**, Data are the mean $\pm$ s.d. **c,f**, P values were determined using an unpaired, two-tailed Student's t -test. **h,j,m**, P values were determined using a log-rank (Mantel–Cox) test.



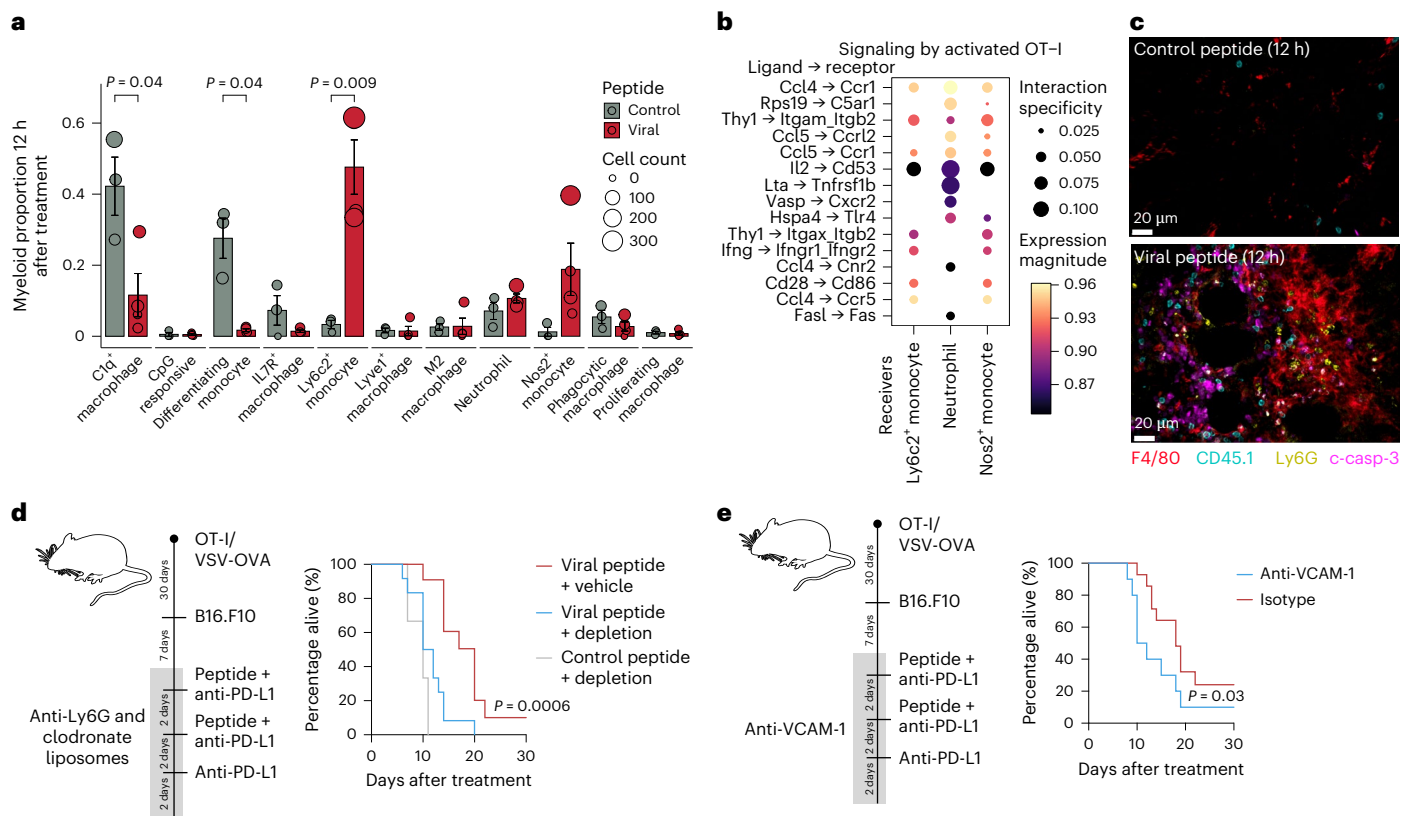


Fig. 5 | VCAM-1 and myeloid cells are essential. **a**, Proportions of myeloid cell clusters across experimental conditions (one CITE-seq experiment; $n = 2$ –4 mice per group). **b**, Predicted ligand–receptor interactions between activated virus-specific memory CD8⁺ T cells and Nos2⁺ or Ly6c2⁺ monocytes and neutrophils, identified using LIANA. **c**, Localization of F4/80⁺, CD45.1⁺, Ly6G⁺ and cleaved caspase 3⁺ cells in B16 melanoma tumors 12 h after control (top) or viral (bottom) peptide; representative images from $n = 3$ mice per group across two independent experiments. **d**, Myeloid cell depletion schematic (left) and survival (right) of B16.F10 tumor-bearing OT-I chimeras treated with viral peptide plus clodronate liposomes and anti-Ly6G (blue, $n = 12$, two experiments), viral peptide plus PBS liposomes and isotype antibody (red, $n = 11$, two experiments),

or control peptide plus clodronate liposomes and anti-Ly6G (gray, $n = 3$, one experiment). Liposomes (200 μl, intravenous) and anti-Ly6G (100 μg, intraperitoneal) were given 24 h before treatment initiation and every other day until endpoint. **e**, VCAM-1 neutralization schematic (left) and survival (right) of B16.F10 tumor-bearing OT-I chimeras treated with anti-VCAM-1 (blue, $n = 10$, two experiments) or isotype antibody (maroon, $n = 14$, two experiments) during peptide treatment; anti-VCAM-1 (500 μg, intraperitoneal) was given 24 h before treatment initiation and every other day until day 6. Data are the mean $\pm$ s.e.m. **a**, P values were determined using an unpaired two-tailed Student's t -test. **d**, P values were determined using a log-rank (Mantel–Cox) test. **e**, P values were determined using a Gehan–Breslow–Wilcoxon test.

be emphasized that treatment required multiple mechanisms, all triggered by activation of T cells, and requiring a combination of effector molecules to recapitulate the response observed in vivo.

It is curious that T cell reactivation largely avoids damaging healthy tissue, suggesting that the mechanism primarily kills cells that have deviated from homeostasis. The mechanism underlying the difference in sensitivity to paracrine killing between melanoma cells and melanocytes remains unclear; however, this may stem from shared features between virally infected cells and neoplastic cells that render them susceptible to this type of programmed cell death. Relevant features may include more rapid cell turnover, a suppressed cell damage response or altered metabolic pathways that are required to overcome oxidative stress^{31,32}. A recently proposed mechanism of T cell-mediated paracrine killing selectively eliminates infected cells while sparing uninfected healthy cells³³. This selectivity arises from microbial factors that inhibit downstream TNFR1 signaling in infected cells, thereby sensitizing them to paracrine cytokine-mediated killing. Perhaps the adaptive immune system exploits these mechanisms to target responses against abnormal cells while sparing healthy tissue. This invites reconsiderations about the specificity of adaptive immunity. While specific lymphocyte antigen recognition is required to initiate the response, the execution of the response may ultimately function through the conduit of many nonspecific cells

who do the heavy lifting. Indeed, stromal and parenchymal cells respond to antipathogen T cell reactivation¹⁰. We may be tapping into a conserved immune mechanism evolved to enable tissue-wide involvement in the eradication of diseased cells. However, we should emphasize that there is exquisite specificity of location (that is, when peptide is injected into healthy skin adjacent to the tumor, the tumors do not regress).

The mechanism underlying the requirement of PD-L1 blockade remains unclear and may depend on PD-L1 expression on either tumor or host cells. Because anti-PD-L1 increased ISG and *Tnf* expression on Ly6c2-expressing monocytes, it is possible that the role of PD-L1 blockade in the viral peptide response is via amplification of innate immune cell activation and effector cytokine production. The overall effect of anti-PD-L1 could be to clear the threshold of activation to mediate complete clearance via cytokine-induced tumor cell death versus transient control. Definitively addressing this question may require a panoply of targeted genetic KO approaches to determine which PD-L1-expressing cell types are responsible for the therapeutic benefit of PD-L1 blockade.

T cell immunotherapies, whether by checkpoint-blockade-mediated manipulation of T cell signaling (PD-1, PD-L1, CTLA-4), next-generation tumor vaccines or adoptive cell therapies, are all thought to depend on tumor specificity. This requires identification of

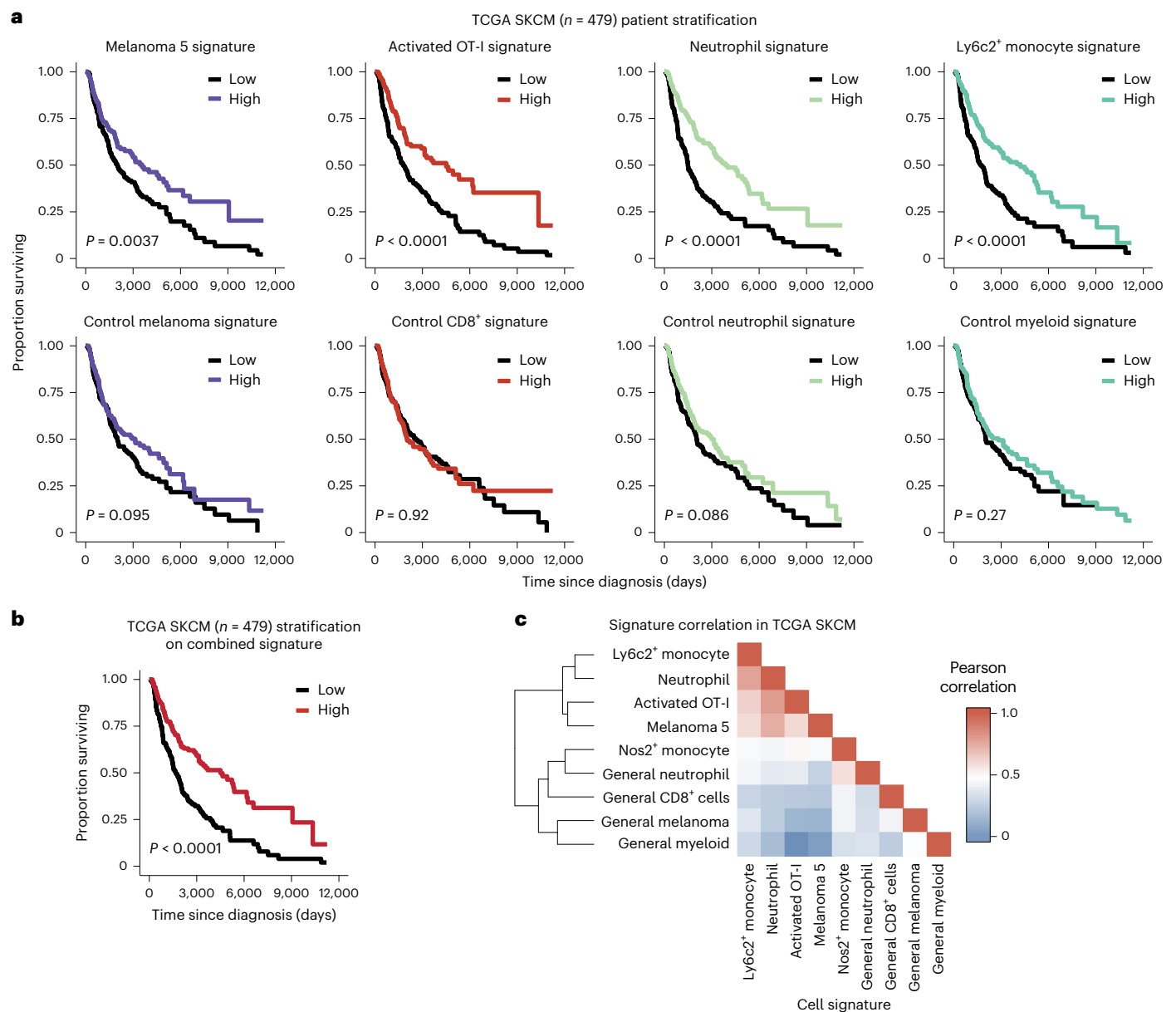


Fig. 6 | Tumoricidal signatures in mice are predictive of survival in patients with melanoma. a, b, Kaplan–Meier survival curves of TCGA patients with skin cutaneous melanoma (SKCM) (n = 479) stratified according to high or low score for each individual gene signature (**a**) or for the combined signature, calculated as the average of the individual signatures (**b**). Exact P values for comparisons

shown as P < 0.0001: activated OT-I, P = 7×10^{-7} ; neutrophil, P = 9.92×10^{-6} ; Ly6c2 monocyte, P = 2.14×10^{-6} ; combined, P = 1.25×10^{-7} . **c,** Pearson's correlation of gene signature scores across patients with SKCM. **a, b,** P values were determined using a two-sided log-rank test.

immunogenic tumor antigens, which are often patient-specific and rare in many cancers, or the putative potentiation of endogenous ineffective tumor-specific T cell responses. Our results suggest that methods that induce robust T cell activation within tumors, regardless of specificity, could be used to effect tumor control. This would substantially decrease the challenges of broadly applying T cell immunotherapy for the treatment of cancer.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41590-026-02642-z>.

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Methods

Mice

Female 6–8-week-old specific-pathogen-free C57BL/6J mice (CD45.2⁺; B6) were obtained from The Jackson Laboratory. Six-week-old female TCR α -deficient mice (B6.129S2-Tcr^{tm1Mom}/J; strain no. 002116) were likewise sourced from The Jackson Laboratory and housed under specific-pathogen-free conditions at the University of Minnesota. CD45.1⁺Thy1.2⁺ OT-I TCR transgenic mice, along with a line generated by crossing these OT-I mice onto a perforin-1-deficient B6 background (C57BL/6-Prf1^{tm1Sdz}/J; strain no. 002407; a gift from the Blazar laboratory), were bred and maintained in-house. Every procedure adhered to the guidelines of the Institutional Animal Care and Use Committee at the University of Minnesota.

Adoptive cell transfer and infection

To establish OT-I immune chimeras, 5×10^4 naive CD45.1⁺Thy1.2⁺ OT-I CD8⁺ T cells isolated from female donors were transferred into female B6 recipients aged 6–8 weeks. The following day, recipients were challenged intravenously with 1×10^6 plaque-forming units of VSV-OVA.

Tumor model

The B16.F10 and B16.F10.ZsG melanoma lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (SH30027.LS, Hyclone) supplemented with 10% FCS (cat. no. FP-0500, Atlas Biologicals), 2×10^{-4} M glutamine (cat. no. 25030-081, Gibco), 10 mM HEPES (cat. no. 15630-080, Gibco), 1 mM sodium pyruvate (cat. no. 11360-070, Gibco), 1×10^{-3} M nonessential amino acids (cat. no. 11140-050, Gibco), β -mercaptoethanol at $1:2,000$ ($1,000\times$, cat. no. 21985-023, Gibco), plasmocin at $5 \mu\text{g ml}^{-1}$ (cat. no. ant-mpp, InvivoGen) and 100 U ml^{-1} penicillin–streptomycin. Cells were dissociated from culture with TrypLE Express (cat. no. 12605-028, Gibco). For the primary tumor studies, 2×10^5 B16 cells were delivered intradermally into the right flank of OT-I chimeric mice. Mice reached endpoint when tumors exceeded 10 mm in both length and width (approximately $>100 \text{ mm}^2$) or when ulceration was accompanied by signs of moribund distress as permitted by the Institutional Animal Care and Use Committee protocol.

CRISPR–Cas9-mediated generation of an H-2K^b KO B16 cell line

B16.F10 cells were cultured as described above. Ribonucleoprotein (RNP) complexes were assembled at room temperature by combining TrueCut Cas9 protein (cat. no. A36499, Thermo Fisher Scientific) with 100 μM gRNA (ACGAAUACCUCAGCGAGUG, Synthego, targeting the H2-K1 gene at position 33999996) at a 1.5:3 ratio. A total of 1×10^6 B16.F10 cells were prepared according to the manufacturer's instructions using the P3 Primary Cell 4D-Nucleofector X Kit S (cat. no. V4XP-3032, Lonza) and combined with the RNP complex. Electroporation was performed on a Lonza 4D-Nucleofector Core X Unit with program DJ-110. Cells were permitted to recover in culture for 7 days, after which recombinant mouse IFN γ (cat. no. 485-MI-100, R&D Systems) was added to a final concentration of 10 ng ml^{-1} . After 48 h of IFN γ exposure, live (Ghost Dye Red 780, Tonbo Biosciences), H-2D^b (KH95, cat. no. 743538, BD Biosciences) and H-2K^b (AF6-88.5, cat. no. 562831, BD Biosciences) cells were isolated on a FACS Aria (BD Biosciences). After a further 6 days of expansion, single cells were seeded per well by limiting dilution. The recovered clones were rescreened after 48 h of IFN γ stimulation to identify populations that were uniformly H-2K^b-. As a control, an aliquot of the parental B16.F10 cells was processed in parallel with an RNP carrying a non-murine-targeting gRNA (Negative Control gRNA no. 1, Synthego); intact expression of both H-2K^b and H-2D^b was confirmed in this line before use. This 'WT gRNA B16' line served as the direct comparator for all experiments involving the H-2K^b KO B16 cell line.

Tissue processing and flow cytometry

For the flow cytometry analyses, inguinal LNs (tumor-draining and non-tumor-draining) were collected and placed in RPMI 1640

supplemented with 5% FCS. LNs were mechanically punctured with fine-tipped forceps, followed by enzymatic digestion in 4 mg ml^{-1} collagenase type IV (cat. no. C6885, Sigma-Aldrich) for 30 min at 37°C . Samples were then passed through a $70\text{-}\mu\text{m}$ cell strainer (cat. no. 352235, Falcon) to obtain single-cell suspensions. B16 tumors were excised, weighed, minced and dissociated using gentleMACS C tubes according to the manufacturer's protocol. Tumor suspensions were filtered through a $70\text{-}\mu\text{m}$ strainer (cat. no. 352350, Corning) before downstream analyses. Surface staining was performed for 1 h at room temperature in Dulbecco's PBS (cat. no. SH30028.03, Hyclone) containing 2% BSA (cat. no. A1009, Sigma-Aldrich) and 1% sodium azide. Intracellular staining was conducted using the Transcription Factor Staining Buffer Kit (cat. no. TNB-1213-L150, Tonbo Biosciences) according to the manufacturer's instructions. Absolute cell counts were determined using PKH26 reference microbeads (cat. no. P7458, Sigma-Aldrich). Tumor-infiltrating monocytes/macrophages were identified as CD11b⁺Ly6C⁺Lineage F4/80⁺ cells, and DCs as Ly6C⁺Lineage⁺MHC-II⁺CD11c⁺CD103⁺ cells. In inguinal LNs, monocytes and macrophages were defined as CD11b⁺Ly6C⁺Lineage F4/80⁺, and DCs as Ly6C⁺Lineage⁺MHC-II⁺CD11c⁺CD103⁺. Migratory DCs were gated as MHC-II^{hi}CD11c^{mid/lo}, resident DCs as MHC-II^{mid/lo}CD11c^{hi}. Lineage markers included B220, Ly6G and Thy1.2 (CD90.2). For the neutrophil depletion experiments, tumor suspensions were intracellularly stained with anti-Ly6G in addition to surface lineage markers. Tumor-infiltrating neutrophils were identified as Ly6G⁺ live singlets.

All antibodies are anti-mouse and were used at 1:100 dilution unless otherwise indicated. Clone, fluorophore, cat. no. and supplier are given for each: CD4 (GK1.5, BV421, cat. no. 562891, BD Biosciences); CD8a (53-6.7, BV605, cat. no. 100744, BioLegend); CD11b (M1/70, BUV737, cat. no. 612800, BD Biosciences); CD11c (N418; AF488, cat. no. 117311, BioLegend; BV650, cat. no. 117339, BioLegend); CD19 (6D5, BV785, cat. no. 115543, BioLegend); CD25 (PC61.5, PE-Cy7, cat. no. 25-0251-82, Thermo Fisher Scientific); CD44 (IM7, BV785, cat. no. 103059, BioLegend); CD45.1 (1:200 dilution, A20, FITC, cat. no. 35-0453-U500, Tonbo Biosciences); CD45.2 (1:200 dilution, 104, APC, cat. no. 109814, BioLegend); CD62L (MEL-14, BUV737, cat. no. 612833, BD Biosciences); CD64 (X54-5/7.1, AF647, cat. no. 139322, BioLegend); CD69 (1:50 dilution, H1.2F3, BV650, cat. no. 104541, BioLegend); CD86 (GL-1, BV650, cat. no. 105036, BioLegend); CD103 (M290, BV510, cat. no. 563087, BD Biosciences); CD172a (P84, PerCP-eFluor710, cat. no. 46-1721-82, BD Biosciences); CD274/PD-L1 (10F.9G2, PE/Dazzle 594, cat. no. 124324, BioLegend); CD279/PD-1 (29F.1A12, BV605, cat. no. 135220, BioLegend); H-2D^b (1:500 dilution, KH95, BV650, cat. no. 743538, BD Biosciences); H-2K^b (1:500 dilution, AF6-88.5, PerCP-Cy5.5, cat. no. 562831, BD Biosciences); I-A/I-E (1:500 dilution, M5/114.15.2, BV510, cat. no. 107635, BioLegend); IFN γ (1:50 dilution, XMGI.2, PE-Cy7, cat. no. 505826, BioLegend); Ly-6C (AL-21, BV605, cat. no. 563011, BD Biosciences); PE-Cy7, cat. no. 560593, BD Biosciences); Ly-6G (1A8, PerCP-Cy5.5, cat. no. 560602, BD Biosciences); NK1.1 (PK136, BUV395, cat. no. 564144, BD Biosciences); PE, cat. no. 108708, BioLegend); Siglec-F (E50-2440, BUV395, cat. no. 740280, BD Biosciences); and XCR1 (ZET, PE, cat. no. 148203, BioLegend).

Peptide, cytokine and anti-PD-L1 treatments

After B16 melanoma implantation, tumor growth was monitored until they reached a size of $\sim 9 \text{ mm}^2$, after which they were enrolled in treatment; $0.5 \mu\text{g}$ of control/irrelevant peptide (gp33-41, KAVYNFATM, Biosynth) or relevant viral peptide (Ova257-264, SIINFELK, Biosynth) was injected into tumors in a volume of $30 \mu\text{l}$ PBS. For the efficacy studies, an additional intratumoral peptide injection was administered 48 h after the first treatment; three $200\text{-}\mu\text{g}$ anti-PD-L1 (clone 10 F.9G2, cat. no. BE0101, Bio X Cell) doses were delivered intravenously on days 0, 2 and 4 after the peptide injections. For the cytokine injections, $1 \mu\text{g}$ of recombinant mouse IFN γ (cat. no. 485-MI-100/CF, R&D Systems), $0.5 \mu\text{g}$ of recombinant mouse TNF (cat. no. 410-MT-050/CF, R&D Systems), and

1 µg of SNAP (cat. no. N3398, Sigma-Aldrich) was injected into tumors in a volume of 50 µl PBS. For the efficacy studies, an additional injection was administered 48 h after first treatment.

Surgical resection of B16 melanoma and LN metastasis identification

Mice were treated with peptide and anti-PD-L1 as described above. One day after the final dose of treatment, tumors were surgically resected as follows. Mice were anesthetized with isoflurane. The tumor and tumor-adjacent skin was shaved and disinfected before incisions along the periphery of the tumor were made and the tumor was released from the subcutaneous adipose tissue using small scissors. Continuous surgical staples were placed to join the skin. After primary B16 tumor resection, mice were euthanized at moribund endpoints or 60 days and dissected to assess the metastases.

Cell depletion and cytokine blockade

NK cell depletion was accomplished with anti-mouse NK1.1 antibody (PK136, cat. no. BE0036, Bio X Cell). Five hundred micrograms of αNK1.1 was injected intraperitoneally 48 h before treatment initiation. Subsequent doses of 200 µg were administered every 48 h up to a total of eight doses. Monocyte depletion was accomplished with intravenous clodronate liposome injections (Liposoma). PBS liposomes were used in control groups. Relative to tumor treatment start, 200 µl of clodronate liposomes was administered on days -1, 0, 2, 4 and 6. Neutrophil depletion was accomplished with anti-Ly6G (1A8-CP129, cat. no. CP129, Bio X Cell). Relative to tumor treatment start, 500 µg of αLy6G was administered intraperitoneally on days -1, 0, 2, 4 and 6. For the cytokine blockade experiments, anti-IFNγ (XMGI.2, cat. no. BP0055, Bio X Cell) or anti-TNF (XT3.11, cat. no. BP0058, Bio X Cell) were administered at a dose of 500 µg on days -1, 1, 3, 5, 7, 9 and 11 after treatment start. VCAM-1 neutralization was accomplished with anti-VCAM-1 antibody (M/K-2.7, cat. no. BE0027, Bio X Cell). Relative to tumor treatment start, 500 µg of antibody was administered on days -1, 0, 2, 4 and 6. Isotype antibodies were used in all control groups as appropriate (anti-HRP, IgG1, HRPN, cat. no. BE0088, Bio X Cell; IgG2a, C1.18.4, cat. no. BE0085, Bio X Cell; anti-Trinitrophenol, 2A3, cat. no. BE0089, Bio X Cell).

Immunofluorescence microscopy

B16 tumors were fixed in 2% paraformaldehyde in PBS for 12–48 h at 4 °C and then dehydrated in 30% sucrose overnight at 4 °C before being frozen in Tissue-Tek O.C.T.; 20-µm sections were cut using a Leica Cryostat and mounted onto Superfrost Plus Microscope Slides (cat. no. 1255015, Fisherbrand). Sections were subsequently stained with fluorescently conjugated antibodies against Ly6G, CD45.1, F4/80 and cleaved caspase 3. Images were acquired with a ×20 immersion objective and a voxel size of 0.568 µm on a Leica Stellaris 8 using laser scanning confocal microscopy with a 1-airy unit pinhole. Grouped sequential scans with excitation wavelengths of 405, 440, 498, 550, 594 and 647 nm were used with spectral HyD-S detectors with emission ranges set to 410–425, 445–510, 505–550, 555–585, 600–630 and 655–690 nm, respectively. Images were analyzed using the Imaris software.

Cell isolation for CITE-seq

After being excised, B16 tumors were minced on parafilm and digested in RPMI 1640 containing 5% FCS, 2 mM MgCl₂, 2 mM CaCl₂ (digestion buffer) and 1 mg ml⁻¹ collagenase type IV and 0.5 mg ml⁻¹ DNase I, and incubated at 37 °C with constant shaking. After 30 min, the remaining tissue pieces were gentleMACs-dissociated.

Fluorescence-activated cell sorting for CITE-seq

Single-cell suspensions of B16 melanoma tumors were prepared from OT-I chimeras as described above. Live cells were enriched using the MojoSort Mouse Dead Cell Removal Kit (cat. no. 480157, BioLegend)

and Fc receptors were blocked by incubating cells with anti-mouse CD16/CD32 (2.4G2, cat. no. 70-0161-M001, Tonbo Biosciences) for 10 min on ice. Live-enriched single-cell suspensions were stained with LIVE/DEAD dye (Ghost Dye Red 780, Tonbo Biosciences), fluorescently conjugated antibodies, oligonucleotide-conjugated TotalSeq A antibodies (BioLegend) and TotalSeq A hashtag antibodies. OT-I T cells (LIVE, CD8a⁺ and CD45.1⁺), endogenous polyclonal CD8⁺ (LIVE, CD8a⁺ and CD45.2⁺) and CD4⁺ (LIVE, CD4⁺), NK cells (LIVE, CD8⁻, CD4⁻ and NK1.1⁺), B cells (LIVE, CD8⁻, CD4⁻ and CD19⁺MHC-II⁺), monocytic cells (LIVE, CD8⁻, CD4⁻, NK1.1⁻, CD19⁻ and Ly6C⁺), DCs (LIVE, CD8⁻, CD4⁻, NK1.1⁻, CD19⁻, Ly6C⁻ and CD11c⁺MHC-II⁺), melanoma cells (side scatter area-high) and stromal cells (LIVE, CD45.2⁻ and CD45.1⁻) were sorted into PBS (without Ca or Mg) with EDTA and FCS using a FACSAria II (BD Biosciences).

Single-cell CITE-seq protocol and library preparation

The CITE-seq experiment was performed according to BioLegend's TotalSeq A Antibodies and Cell Hashing with 10× Single Cell 3' Reagent Kit v.3 or v.3.1 (Single Index) protocol. Briefly, sorted cells were loaded onto a 10X Genomics Chromium Controller at a concentration of 1,300–1,600 cells per µl to target a total cell recovery of 10,000–15,000 cells per lane.

Single-cell CITE-seq data preprocessing

For the CITE-seq libraries, CellRanger v.7.1.0 (10X Genomics, genome build GRCm38) was used to align raw sequencing data. The ADT and HTO library sequences were aligned to a custom reference genome provided by BioLegend containing oligonucleotide sequences corresponding to each antibody. Seurat v.5 was used to process the single-cell RNA, ADT and HTO libraries. High-quality cells were identified by filtering for a minimum 200 genes and 800 unique molecular identifiers detected, and a maximum mitochondrial content of 20%. Counts were log₂+1 transformed and normalized to 10,000 counts per cell.

Single-cell CITE-seq cell clustering and annotation

After preliminary filtering, hashed samples were demultiplexed using the standard Seurat workflow with default parameters. The top 2,000 highly variable genes were identified, scaled and used for dimensionality reduction via principal component analysis. An elbow-based selection criterion selected the number of principal components, followed by construction of *k*-nearest-neighbor and shared nearest-neighbor graphs and UMAP visualization. Leiden-based clustering with a resolution chosen via a parameter scan to maximize silhouette identified major clusters for broad-level cell type annotation. Broad lineages were annotated based on canonical RNA and surface protein markers and the CellTypist encyclopedia. Within each lineage, iterative subclustering as described above was performed to annotate granular cell types. Low-quality transcriptomes and doublets were identified by the high expression of mitochondrial genes or well-established low-quality marker genes, and co-expression of mutually exclusive lineage markers, respectively, and were excluded from the analysis. Red blood cells identified using high expression of hemoglobin genes (*Hba-a1*, *Hbb-bt*) were also excluded from the analysis.

Pathway enrichment analysis

To characterize response to treatment across cell types and cell states, differential expression testing was performed and significant genes were defined as those with a *P*_{adj} < 0.05 (Wilcoxon rank-sum test), expressed in more than 25% of cells and an average log(fold change) > 0.5 or ≤ 0.5. To identify pathways enriched in specific treatment groups or cell types, we used the Enrichr pipeline to perform enrichment analysis of differentially expressed genes against gene sets from the Hallmark database and Gene Ontology Biological Pathways database. Signature scores for enriched signatures were calculated using the UCell pipeline.

Cell–cell interaction analysis

To identify putative patterns of differential cell–cell interactions, we used the MultiNicheNet pipeline, leveraging pseudobulk cell type profiles to identify differential, context-dependent communication events. To identify key cell–cell interactions, we used the LIANA framework and consensus ligand–receptor database. To determine drivers of cell state and link them to cell communication, we used the LIANA and NicheNet frameworks. LIANA prioritized a ranked list of ligand–receptor interactions from transcriptomic data, while NicheNet predicts messenger ligands based on a set of transcriptional targets.

Cell abundance analysis

Within sorted broad lineages, the cell type proportion and Spearman correlation coefficients for all pairwise cell type combinations were calculated to identify co-abundant cell types across treatment groups. Then, hierarchical clustering of the coefficients identified five clusters of correlated, co-occurring cell types. To understand the relationship between identified cell type clusters and treatment groups, the abundance of each cluster was examined for each mouse and condition.

Survival analysis

To perform patient stratification analysis, bulk transcriptomic data for patients with SKCM was obtained from the TCGA database. Patients without overall survival data were removed from the analysis. Bulk expression profiles were normalized, $\log_2$ -transformed and used to calculate cell signature scores per sample based on average gene expression. Stratification and visualization was performed using the Rsurvival and survminer packages. Correlations of gene signatures in patients were calculated using Pearson's correlation.

In vitro cytokine treatment and annexin V/PI staining

Recombinant mouse IFN γ and recombinant mouse TNF were reconstituted at 100 $\mu\text{g ml}^{-1}$ in sterile PBS. Animal-free recombinant human IFN γ (cat. no. AF-300-02, PeproTech) and recombinant human TNF (cat. no. 300-01A, PeproTech) were reconstituted in sterile PBS. SNAP was reconstituted at 100 mM in dimethyl sulfoxide. Cells were seeded in a 96-well plate at 10,000 cells per well. Twenty-four hours later, cells were treated with single or combination cytokines. Mouse cell lines were treated with species-matched mouse cytokines and human cell lines with human cytokines, in both cases at 2 $\mu\text{g ml}^{-1}$ IFN γ and 0.1 $\mu\text{g ml}^{-1}$ TNF, with SNAP at 100 μM . Cells were analyzed 20–30 h later. Annexin V and PI staining was performed using the FITC Annexin V Apoptosis Detection Kit I (cat. no. 556547, BD Biosciences) according to the manufacturer's instructions. Briefly, cells were lifted using trypsin, washed with cold PBS, then stained with Annexin V and PI for 15 min at room temperature. Sample data were acquired immediately on an Aurora instrument (Cytex).

Cell lines

B16.F10 mouse melanoma cells were obtained from ATCC (cat. no. CRL-6475; derived from the skin of a C57BL/6 mouse; donor sex not specified by the depositor). YUMM1.7 mouse melanoma cells (cat. no. CRL-3362, ATCC; derived from the skin of an adult male C57BL/6 mouse) were a gift from the Rothlin Lab at the University of Minnesota and cultured in DMEM/F12 with 10% FCS, 1% nonessential amino acids and 100 U ml^{-1} penicillin–streptomycin. SK-MEL-2 human melanoma cells (originally deposited at ATCC as cat. no. HTB-68; established from a cutaneous metastasis of a 60-year-old male patient with malignant melanoma) were a gift from the Freedman laboratory, University of Minnesota, and were cultured in DMEM with 10% FCS 1 mM sodium pyruvate and 100 U ml^{-1} penicillin–streptomycin. A375 human melanoma cells (originally deposited at ATCC as cat. no. CRL-1619; established from the skin of a 54-year-old female patient with malignant melanoma) were a gift from the Costalonga laboratory, University of Minnesota, and were cultured in DMEM with 10% FCS and 100 U ml^{-1} penicillin–streptomycin.

Primary adult human epidermal melanocytes (cat. no. PCS-200-013, ATCC) were cultured in Dermal Cell Basal Medium (cat. no. PCS-200-030, ATCC) supplemented with the Adult Melanocyte Growth Kit (cat. no. PCS-200-042, ATCC). All cells were maintained at 37 °C in 5% CO $_2$.

In vitro reactivated memory T cell supernatant assay

CD8 $^+$ T cells were enriched through negative selection before they were stimulated. CD8 $^+$ T cell enrichment was carried out using a prepared cocktail of biotinylated antibodies. Briefly, single-cell suspensions were resuspended at 10 ^{8} cells per ml in PBS (Gibco) supplemented with 2% heat-inactivated fetal bovine serum (Peak Serum) and 1 mM EDTA (Promega Corporation) and then incubated with 2% Mouse FcR Blocker (STEMCELL Technologies) and 0.0275 mg ml^{-1} anti-mouse CD4 (GK1.5) from Invitrogen and anti-mouse CD19 (1D3), anti-mouse CD11b (M1/70), anti-mouse NK1.1 (PK136), anti-mouse F4/80 (BM8.1), anti-mouse TER119, anti-mouse CD45R (RA3-6B2), anti-mouse LY6G (GR1) and anti-mouse CD16/32 (2.4G2) from Tonbo Biosciences. All antibodies were conjugated to biotin. Antibody-bound cells were removed using magnetic enrichment with the EasySep Mouse Streptavidin RapiidSpheres Isolation kit (STEMCELL Technologies) according to the manufacturer's instructions. Purified CD8 $^+$ T cells were plated at 1 $\times$ 10 ^{6} cells per well in T cell medium supplemented with 2 $\mu\text{g ml}^{-1}$ SIINFEKL or gp33 (control) peptide. After 30 h, supernatants were collected and transferred to B16 cells plated in 96-well plates; 30 h later, the percentage of annexin V $^+$ B16 cells was quantified, as described above.

In vitro death pathway inhibition

Cell lines were treated with recombinant cytokines and SNAP as described above. Death pathway inhibitors were added to cultures concurrently with cytokine treatment. Vehicle controls were treated with dimethyl sulfoxide. RIPK3 inhibition was achieved using GSK'872 (cat. no. S8465, Selleck Chemicals) at 3 μM . Caspase activity was inhibited with zVAD-FMK (cat. no. A1902, ApexBio) at 50 μM . Ferroptosis was inhibited with liproxstatin-1 (cat. no. 11730, Cayman Chemical) at 1 μM .

Paraffin infiltrating, embedding and sectioning for H&E staining

Fixed tissue was immersed in a series of increasing ethanol concentrations (70%, 80%, 95% and 100%) to replace water in the tissue. The tissue was then treated with a clearing reagent, such as xylene, followed by infiltration with several changes of molten paraffin wax to completely replace the clearing reagent. The tissue was placed into a metal mold containing paraffin, oriented carefully at the bottom of the mold, and the cassette was filled and placed on a cold plate for rapid solidification, forming a paraffin block ready for sectioning. Paraffin-embedded tissue blocks were sectioned at 6- μm thickness using a microtome. Sections were floated on a water bath to remove wrinkles, then transferred to clean glass slides, allowed to dry and baked overnight in an oven to ensure adhesion. To visualize cellular structures such as nuclei and cytoplasm, providing a high-contrast view of tissue architecture, sections were stained with H&E. Slides were first deparaffinized in xylene and rehydrated through a graded series of alcohols to water. Sections were stained with hematoxylin for 2–4 min and briefly rinsed, followed by differentiation in acid alcohol and bluing in tap water or bluing buffer to render nuclei blue. Slides were then counterstained with eosin for a shorter duration, dehydrated through graded alcohols, cleared in xylene and mounted with coverslips using a mounting medium.

H&E slide cell death scores

Cell death was quantified in H&E-stained tissue sections by a single unblinded dermatopathologist. Each section was evaluated in its entirety for characteristic morphological features of cell death, including karyorrhexis, pyknosis and the presence of ghost cells and necrotic debris. A score was assigned reflecting the estimated fraction of total tissue area consisting of dead or dying cells.

Statistical methods

No statistical methods were used to predetermine sample size but group sizes are similar to those reported in previous publications¹¹. No randomization was performed. Mice were enrolled only after developing discrete, measurable tumors (~9 mm²); no animals were excluded after treatment initiation except for welfare reasons or technical injection failure. Investigators were not blinded to group allocation during the experiments or outcome assessment. The statistical test, group size (*n*) and *P* values for each experiment are reported in the corresponding figure legend. *P* < 0.05 was considered statistically significant. Data distribution was assumed to be normal but this was not formally tested. Individual data points are shown in all figures.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The CITE-seq data have been deposited in the Gene Expression Omnibus under the accession no. [GSE324375](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE324375). All other data are present in the article and Supplementary Information.

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Author contributions

Conceptualization: O.C.G.S., T.T.D., N.V.G., V.V., A.K.S. and D.M. Methodology: O.C.G.S., T.T.D., N.V.G., A.J.R., S.N., V.J., M.F.K., N.L. and A.G. Investigation: O.C.G.S., N.V.G., S.D.O., M.B.W., E.W., V.J. and A.G. Writing—original draft: O.C.G.S. and D.M. Writing—review and editing: O.C.G.S., T.T.D., N.V.G., S.D.O., M.B.W., A.G.S., P.C.R., V.V., A.K.S. and D.M. Visualization: O.C.G.S., T.T.D. and N.V.G. Supervision: C.F.Q., A.G.S., P.C.R., M.A.G., J.S.M., V.V., A.K.S. and D.M. Funding acquisition: D.M.

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Competing interests

D.M. reports compensation for consulting or scientific advisory roles from Trojan Bio. A.K.S. reports compensation for consulting or scientific advisory board membership from Honeycomb Biotechnologies, Cellarity, Ochre Bio, Relation Therapeutics, Quotient Therapeutics, Wolf Greenfield, Bio-Rad Laboratories, IntrECate Biotherapeutics, Parabilis Medicines, JnJ, Danaher, Passkey Therapeutics and Dahlia Biosciences unrelated to this work. A.K.S. reports research support from Break Through Cancer, Wellcome Leap, NIH, the Bill & Melinda Gates Foundation, Foundation MIT, the Moore Foundation, the Chan Zuckerberg Initiative, Coca-Cola, the UK Medical Research Council, UK Research and Innovation, St. Jude Children's Research Hospital and Becton Dickinson and Company. M.A.G. reports compensation for speaking or board membership from GSK, Fairview and AstraZeneca. M.A.G. reports research support from HCW Biologics and Fate Therapeutics. The other authors declare no competing interests.

Additional information

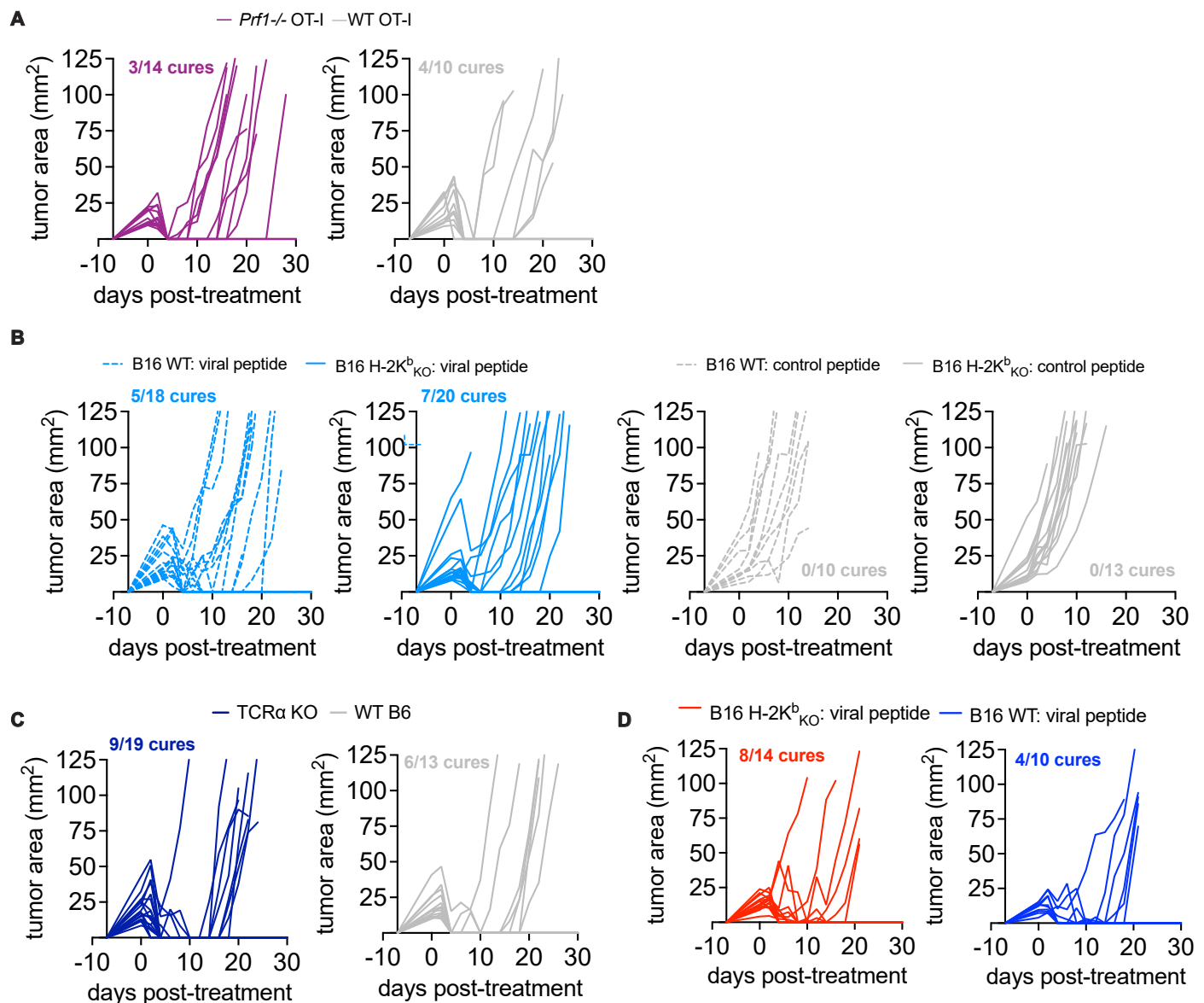
Extended data is available for this paper at <https://doi.org/10.1038/s41590-026-02642-z>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41590-026-02642-z>.

Correspondence and requests for materials should be addressed to David Masopust.

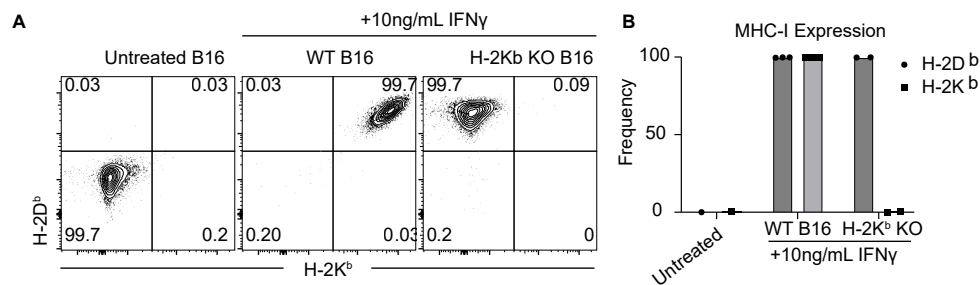
Peer review information *Nature Immunology* thanks Eui-Cheol Shin and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Primary Handling Editor: Nick Bernard in collaboration with the rest of the *Nature Immunology* editorial team.

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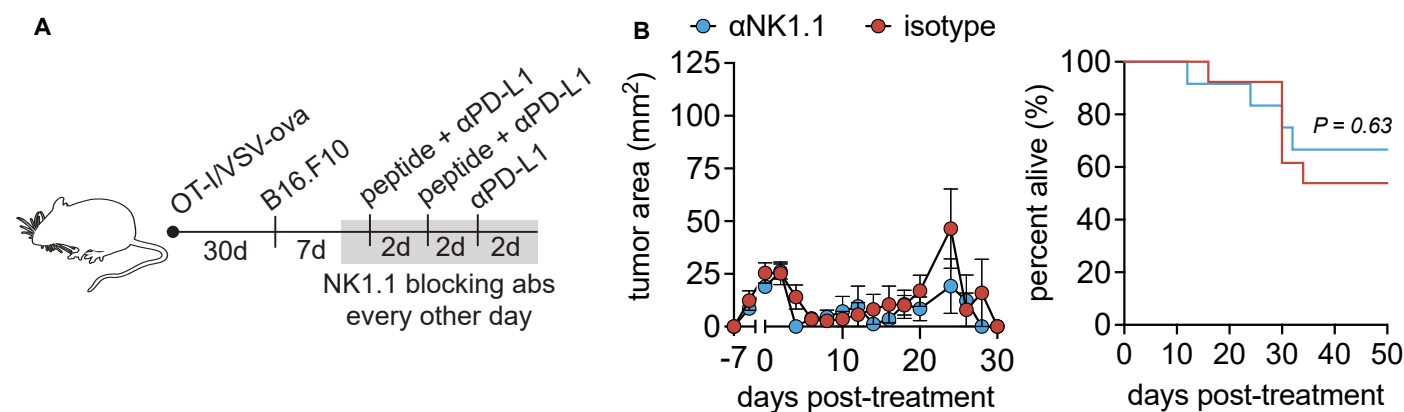
Extended Data Fig. 1 | Tumor growth in individual mice. Tumor growth for individual mice corresponding to the pooled data in Fig. 1c,d (A), Fig. 1g,h (B), Fig. 2h,i (C) and Fig. 2l,m (D). (A) B16.F10 tumor-bearing *Prf1*^{-/-} (purple, *n* = 14) or WT (grey, *n* = 10) OT-I memory mice. (B) B16.F10 tumor-bearing OT-I memory mice given anti-PD-L1 with viral peptide (blue) or irrelevant peptide (grey), bearing H-2K^b KO (solid, *n* = 20 and 13, respectively) or WT (dashed, *n* = 18

and 10) tumors. (C) TCR α KO (*n* = 19) or WT (*n* = 13) OT-I memory mice bearing B16.F10 tumors, treated with viral peptide and anti-PD-L1. (D) TCR α KO OT-I memory mice bearing H-2K^b KO (red, *n* = 14) or WT (blue, *n* = 10) B16.F10 tumors, treated with viral peptide and anti-PD-L1. All data are from two independent experiments.



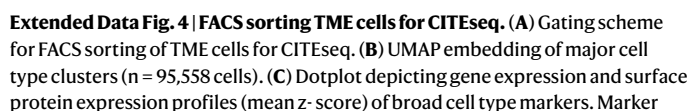
Extended Data Fig. 2 | CRISPR/Cas9 deletion of H-2K^b in B16 melanoma. CRISPR/Cas9 editing and limiting dilution cloning were used to generate an H-2K^b KO B16.F10 melanoma cell line. Loss of H-2K^b expression and retention of H-2D^b expression were confirmed by flow cytometry after 48 h of in vitro

stimulation with recombinant mouse IFN γ (10 ng/mL). **(A)** Representative flow plots showing expression levels of H-2K^b and H-2D^b on B16 cells, quantified in **(B)**. **n** = 1-3 biological replicates (B16 clones) from one experiment.

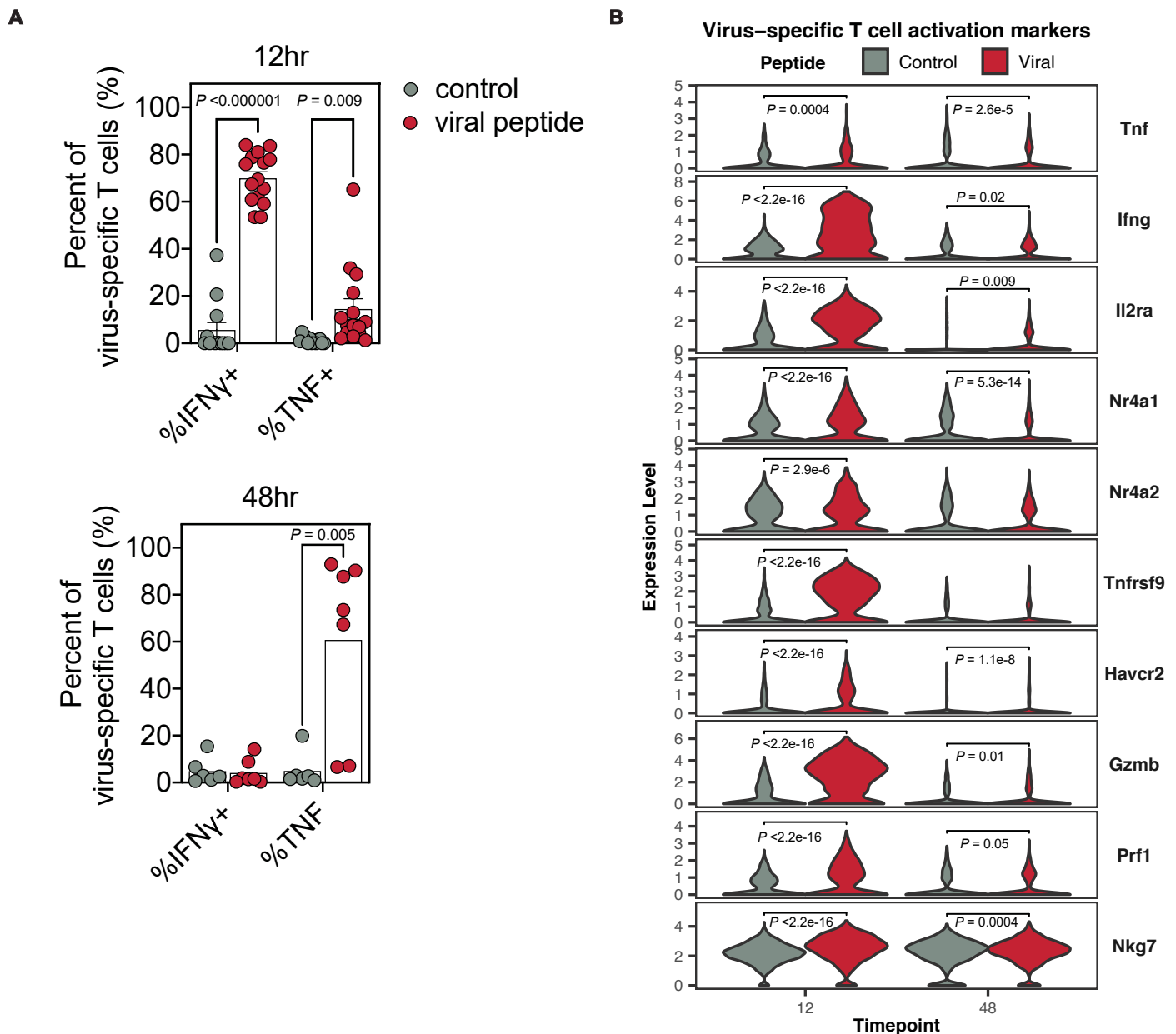


Extended Data Fig. 3 | NK cells are not required. (A) NK cell depletion experimental schematic. (B) Tumor growth (left) and survival (right) of B16.F10 tumor-bearing OT-I chimeras treated with anti-NK1.1 antibody (blue line, $n = 12$ mice across two experiments) or isotype control (red line, $n = 13$ across

two experiments) during peptide treatment. Antibody was administered i.p. at 500 μg 48 h before treatment initiation, followed by 200 μg every other day until endpoint (maximum 8 doses). Tumor growth data are shown as mean $\pm$ SEM. P determined by log-rank Mantel-Cox test for survival.

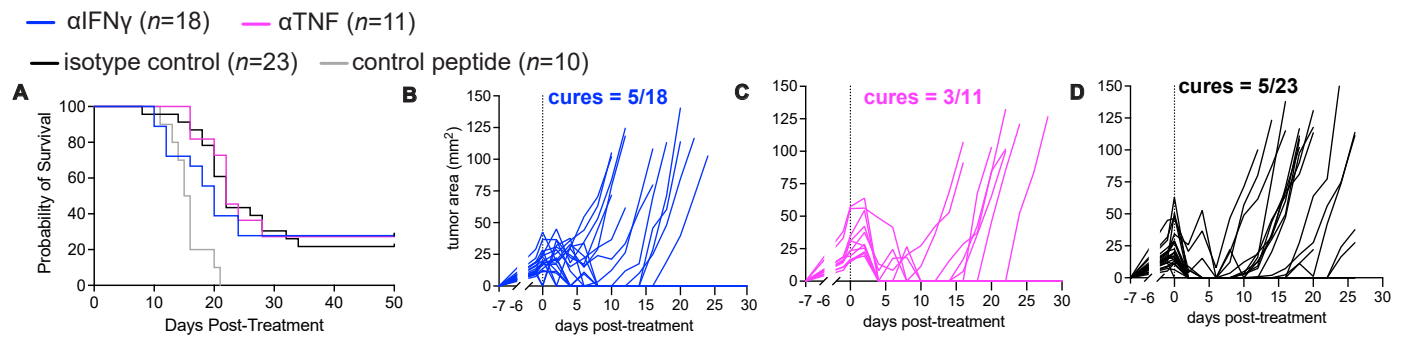


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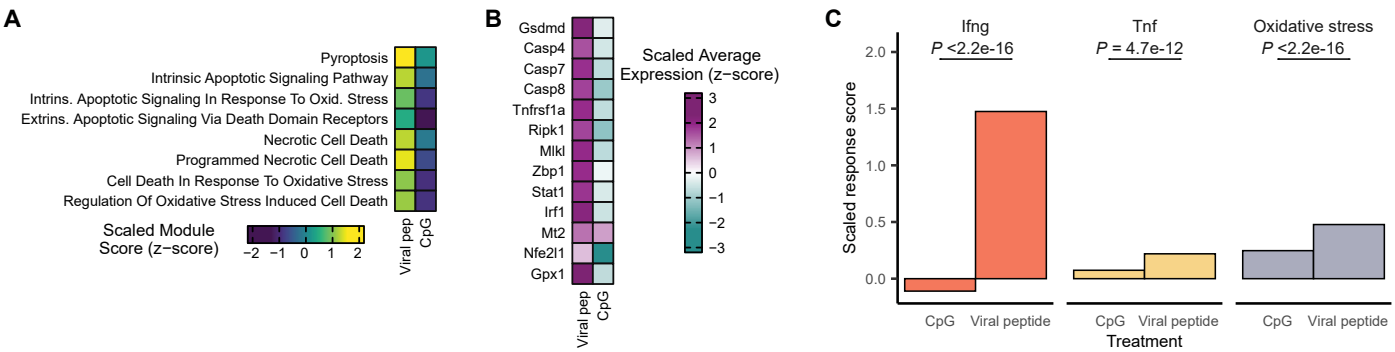


Extended Data Fig. 5 | Virus-specific memory T cell activation. (A) Proportion of IFN γ ⁺ or TNF⁺ OT-I in B16 melanoma tumors 12 h (top) or 48 h (bottom) following intratumoral irrelevant control (grey circles) or viral peptide (red circles) measured by intracellular cytokine staining. (2-4 experiments; $n = 13$ mice treated with irrelevant peptide, $n = 15$ mice treated with viral peptide). Data are

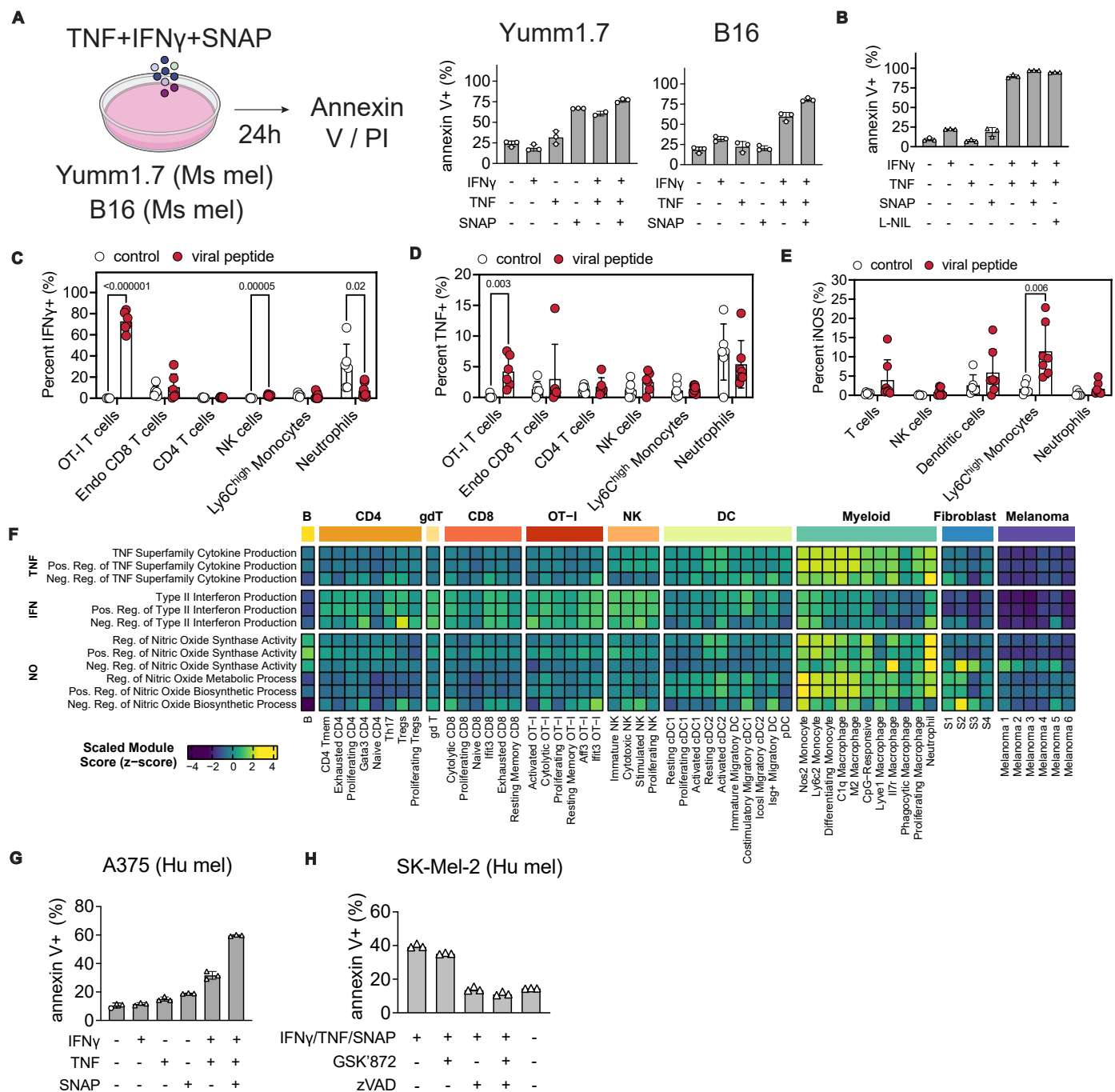
shown as mean $\pm$ SEM and P value as determined by unpaired two-tailed Student's t test. (B) Expression of selected genes by OT-I in B16 melanoma tumors at indicated timepoints following intratumoral control (grey) or viral peptide (red) measured by CITEseq. P was determined by unpaired two-tailed Student's t test.



Extended Data Fig. 6 | Blockade of single cytokines does not prevent tumor clearance. (A) Survival and (B–D) tumor growth of mice treated with anti-IFN γ (XMG1.2) (blue, $n = 18$ mice, two experiments), anti-TNF (XT3.11) (pink, $n = 11$ mice, one experiment) or isotype control (black, $n = 23$ mice, two experiments) during viral peptide + anti-PD-L1 therapy.

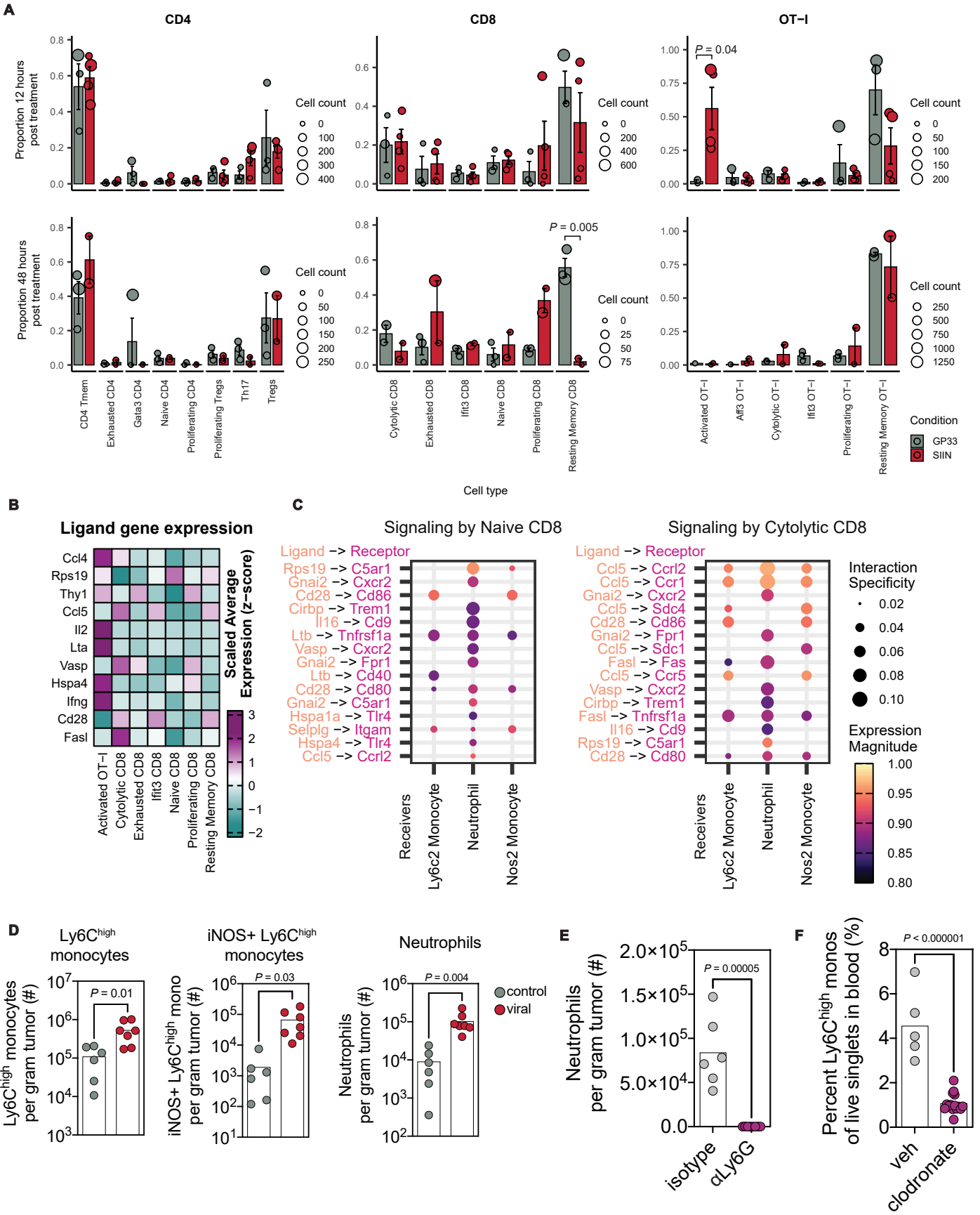


Extended Data Fig. 7 | Melanoma cell response to CpG. (A–B) Expression of programmed cell death pathways **(A)** and selected genes **(B)** in melanoma cells across treatment groups. **(C)** Scaled cytokine response signature in CpG vs viral peptide treated melanoma cells. P was determined by unpaired two-tailed Student’s t test.



Extended Data Fig. 8 | Reactivated virus-specific CD8 + T cells trigger caspase-dependent tumor cell death through cytokines and nitric oxide. (A) In vitro cytokine treatment: schematic (left) and proportion of Annexin V+ YUMML7 or B16 cells (right) after indicated treatments (n = 3 technical replicates from one experiment). Data are shown as mean $\pm$ SEM. (B) Proportion of Annexin V + B16 cells after indicated treatments (n = 3 technical replicates from one experiment). (C-E) Proportion of IFN γ + (C), TNF+ (D), and iNOS+ (E) cells across indicated immune cell types in B16 melanoma 12 h after irrelevant control (white dots)

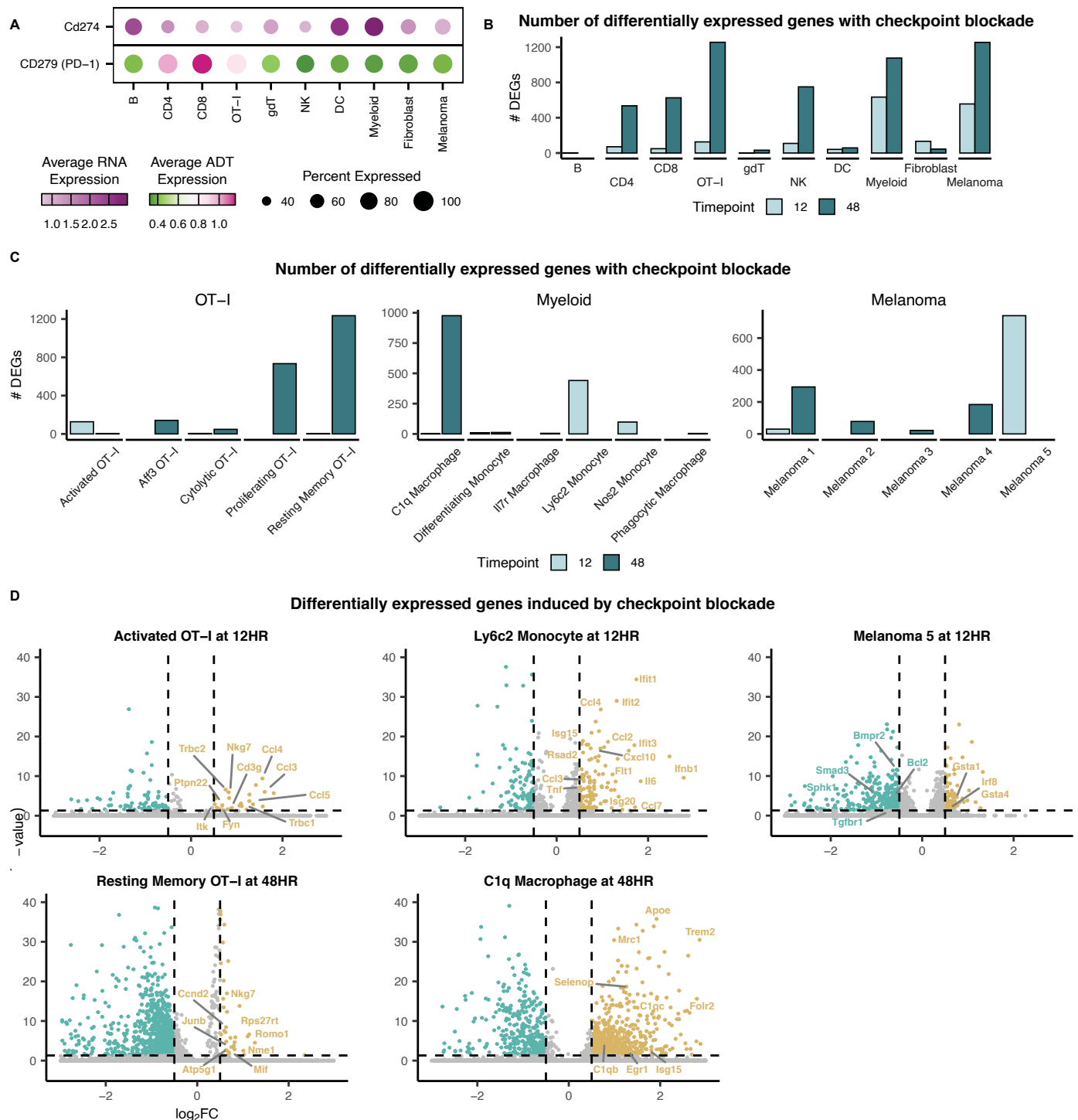
or viral peptide (red dots) (two experiments; n = 6 mice treated with irrelevant peptide, n = 7 mice treated with viral peptide). Data are shown as mean $\pm$ SEM and P value as determined by unpaired two-tailed Student's t test. (F) Expression of TNF, type II interferon and NO production pathways, derived from the Gene Ontology Biological Processes database, across all cell types. (G) Proportion of Annexin V + A375 cells after indicated treatments. (H) Proportion of Annexin V + SK-MEL-2 cells treated with IFN γ +TNF+SNAP plus indicated inhibitors (n = 3 technical replicates across one experiment). Data are shown as mean $\pm$ SEM.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Reactivated virus-specific T cells trigger myeloid cell recruitment. (A) Proportions of T cell states within major subsets, endogenous CD4⁺ (left), endogenous CD8⁺ (middle) and adoptively transferred OT-I (right), across treatment conditions at indicated timepoints post-treatment. P was determined by unpaired two-tailed Student's t test. (B) Gene expression of the top Activated OT-I signaling ligands in the endogenous CD8⁺ T population. (C) predicted ligand–receptor interactions between Naive CD8⁺ T cells (left) or Cytolytic CD8⁺ T cells (right) and Nos2⁺ or Ly6c2⁺ monocytes and neutrophils, identified using LIANA. (D) Quantification of indicated cell types per gram of B16 tumor 12 h after treatment with irrelevant control (grey dots) or viral peptide

(red dots). (E) Quantification of neutrophils per gram of B16 tumor 12 h after viral peptide + anti-PD-L1 therapy in mice treated with anti-Ly6G or isotype control antibodies as shown in Fig. 5d (two experiments; n = 6 mice treated with isotype control, n = 8 mice treated with anti-Ly6G). (F). Proportion of Ly6Chigh monocytes of live singlets in the blood of mice 12 h after viral peptide + anti-PD-L1 therapy in mice treated with clodronate liposomes or PBS-loaded liposomes (veh) as shown in Fig. 5d (one experiment; n = 5 mice treated with veh, n = 15 mice treated with clodronate liposomes). Data are shown as mean ± SEM and P was determined by unpaired two-tailed Student's t test.



Extended Data Fig. 10 | Anti-PD-L1 therapy with viral peptide treatment alters gene expression patterns in key cell types. (A) Dotplot depicting the average gene expression of Cd274 and surface protein expression of PD-1 across broad cell types. (B) Number of significant differentially expressed genes with checkpoint blockade for each broad cell type, colored by time point (C) Number of significant differentially expressed genes with checkpoint blockade within the

OT-I, myeloid and melanoma compartments, colored by time point. (D) Volcano plot highlighting significantly upregulated and downregulated genes (absolute value of average log fold change > 0.5, adjusted p-value < 0.05) in response to viral peptide and checkpoint blockade as compared to viral peptide only treatment.

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| n/a | Confirmed |
| ☐ | ☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☒ | ☐ A description of all covariates tested |
| ☐ | ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
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| ☐ | ☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☒ | ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☐ | ☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐ | ☒ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	No software was used for data collection.
Data analysis	Transcriptomics data was processed via the Cumulus V2.4.0 pipeline and analyzed in R (4.4.0) using Seurat (v.5), UCell (2.10.1), survminer (0.5.0), survival (3.7.0), EnrichR (4.3), Liana (0.1.14), Fgsea (1.32.0) and Ggsignif (0.6.4). GraphPad Prism7, Flowjo version 10.4.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

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 - A description of any restrictions on data availability
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All sequencing data generated during this study will be publicly available through the NCBI Gene Expression Omnibus (GEO) via the accession number GSE324375.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

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Use the terms *sex* (biological attribute) and *gender* (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.

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Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status). Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.) Please provide details about how you controlled for confounding variables in your analyses.

Population characteristics

Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

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Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight

Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical methods were used to predetermine sample size. Sample sizes were selected based on prior experience with the experimental models and historical variability in treatment response. Group sizes were chosen to detect biologically meaningful differences between experimental conditions. For survival experiments, cohort sizes were determined based on historical cure rates and variability in this model to allow detection of differences in survival between groups.

Data exclusions

Mice were enrolled into treatment cohorts only after developing discrete, measurable melanoma tumors. Enrollment occurred when tumors reached approximately 9 mm² in surface area. Mice that failed to develop palpable tumors were not included in treatment or subsequent analyses. No animals were excluded from analysis after treatment initiation unless required for animal welfare reasons or technical intratumoral injection failure.

Replication

All key findings were independently replicated in at least two independent experiments unless otherwise indicated in the figure legends. For mouse tumor studies, each experiment consisted of independently infected (VSV) and then independently inoculated (B16) cohorts of mice and was performed on separate occasions. Biological replicates were defined as individual mice. Technical replicates were defined as individual wells of cells treated independently within the same experiment. No experiments were excluded from analysis due to failure to replicate.

Randomization

No randomization was done during group allocations. Animals of similar age and sex (littermates where possible) were used to control for covariates.

Blinding

Investigators were not blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

FACS

All antibodies were used at 1:100 unless otherwise indicated.
 anti-mouse CD16/CD32 (clone 2.4G2) – Tonbo Biosciences – 70-0161-M001
 anti-mouse NK-1.1, PE (clone PK136) – BioLegend – 108708
 anti-mouse CD19, BV785 (clone 6D5) – BioLegend – 115543
 anti-mouse Ly-6C, PE-Cy7 (clone AL-21) – BD Pharmingen – 560593
 anti-mouse CD11c, BV650 (clone N418) – BioLegend – 117339
 anti-mouse I-A/I-E, BV510 (clone M5/114.15.2, 1:500) – BioLegend – 107635
 anti-mouse CD4, BV421 (clone GK1.5) – BD Pharmingen – 562891
 anti-mouse CD45.2, APC (clone 104, 1:200) – BioLegend – 109814
 anti-mouse CD45.1, FITC (clone A20, 1:200) – Tonbo Biosciences – 35-0453-U500
 anti-mouse CD8a, BV605 (clone 53-6.7) – BioLegend – 100744
 anti-mouse CD44, BV785 (clone IM7) – BioLegend – 103059
 anti-mouse IFN- γ , PE/Cy7 (clone XMG1.2, 1:50) – BioLegend – 505826
 anti-human/mouse Granzyme B, PE/Dazzle 594 – BioLegend – 372216
 anti-mouse CD279 (PD-1), BV605 (clone 29F.1A12) – BioLegend – 135220
 anti-mouse CD274 (PD-L1), PE/Dazzle 594 (clone 10F.9G2) – BioLegend – 124324
 anti-mouse CD11c, AF488 (clone N418) – BioLegend – 117311
 anti-mouse CD86, BV650 (clone GL-1) – BioLegend – 105036
 anti-mouse CD69, BV650 (clone H1.2F3) – BioLegend – 104541
 anti-mouse CD103, BV510 (clone M290) – BD – 563087
 anti-mouse Ly6G, PerCP-Cy5.5 (clone 1A8) – BD – 560602
 anti-mouse CD11b, BVU737 (clone M1/70) – BD – 612800
 anti-mouse NK1.1, BVU395 (clone PK136) – BD – 564144
 anti-mouse Ly6C, BV605 (clone AL-21) – BD – 563011
 anti-mouse CD25, PE-Cy7 (clone PC61.5) – Thermo Scientific – 25-0251-82
 anti-mouse CD62L, BVU737 (clone MEL-14) – BD – 612833
 anti-mouse CD64 (Fc γ RI), AF647 (clone X54-5/7.1) – BioLegend – 139322
 anti-mouse XCR1, PE (clone ZET) – BioLegend – 148203
 anti-mouse Siglec-F, BVU395 (clone E50-2440) – BD – 740280
 anti-mouse CD172a, PerCP-eFluor710 (clone P84) – BD – 46-1721-82
 anti-mouse H-2Db (clone KH95), BV650 – BD Biosciences – 743538
 anti-mouse H2-Kb (clone AF6-88.5), PerCP-Cy5.5 – BD Biosciences – 562831

Blockade Antibodies

(antibody (clone) - Vendor - Identifier)
 anti-mouse CD106 (VCAM-1), InVivoMab – Bio X Cell – BE0027
 anti-mouse IFN γ , InVivoPlus – Bio X Cell – BP0055
 anti-mouse TNF α , InVivoPlus – Bio X Cell – BP0058
 anti-mouse Ly6G, RecombiMab – Bio X Cell – CP129
 anti-mouse IgG2a isotype control, InVivoPlus – Bio X Cell – BP0085
 anti-mouse PD-L1 (B7-H1), InVivoMab – Bio X Cell – BE0101

Immunofluorescence

(antibody (clone) - Vendor - Identifier)
 anti-mouse cleaved caspase 3 – R&D Systems (Bio-Techne) – MAB835
 anti-mouse F4/80, AF594 (clone BM8) – BioLegend – 123140
 anti-mouse CD45.1, BV480 (A20) – BD Biosciences – 746666

CITE-seq / Feature-barcoding antibodies

(antibody - clone - sequence)
 anti-mouse CD4 – RM4-5 – AACAAAGACCTTGAG
 anti-mouse CD8a – 53-6.7 – TACCCGTAATAGCGT
 anti-mouse CD366 (Tim-3) – RMT3-23 – ATTGGCACTCAGATG
 anti-mouse CD279 (PD-1) – RMP1-30 – GAAAGTCAAAGCACT

anti-mouse Ly-6C – HK1.4 – AAGTCGTGAGGCATG
 anti-mouse/human CD11b – M1/70 – TGAAGGCTCATTTGT
 anti-mouse Ly-6G – 1A8 – ACATTGACGCAACTA
 anti-human/mouse CD49f – GoH3 – TTCCGAGGATGATCT
 anti-mouse/human CD44 – IM7 – TGGCTTCAGGTCCTA
 anti-mouse CD54 – YN1/1.7.4 – ATAACCGACACAGTG
 anti-mouse CD90.2 – 30-H12 – CCGATCAGCCGTTTA
 anti-mouse CD73 – TY/11.8 – ACACTTAACGTCTGG
 anti-mouse CD49d – R1-2 – CGCTTGACGCTTAA
 anti-mouse CD200 (OX2) – OX-90 – TCAATTCCGGTAGTC
 Mouse IgG1, κ isotype Ctrl – MOPC-21 – GCCGGACGACATTAA
 Mouse IgG2a, κ isotype Ctrl – MOPC-173 – CTCCTACCTAAACTG
 Mouse IgG2b, κ isotype Ctrl – MPC-11 – ATATGTATCACGCGA
 anti-mouse CD19 – 6D5 – ATCAGCCATGTCACT
 Rat IgG2b, κ isotype Ctrl – RTK4530 – GATTCTTGACGACCT
 anti-mouse CD45 – 30-F11 – TGGCTATGGAGCAGA
 anti-mouse CD25 – PC61 – ACCATGAGACACAGT
 anti-mouse/human CD45R/B220 – RA3-6B2 – CCTACACCTCATAAT
 anti-mouse CD102 – 3C4 (MIC2/4) – GATATTCAGTGCGAC
 anti-mouse CD115 (CSF-1R) – AFS98 – TTCCGTTGTTGTGAG
 anti-mouse CD11c – N418 – GTTATGGACGCTTGC
 anti-mouse CD21/CD35 (CR2/CR1) – 7E9 – GGATAATTTTCGATCC
 anti-mouse CD23 – B3B4 – TCTCTTGAAGATGA
 anti-mouse CD43 – S11 – TTGGAGGGTTGTGCT
 anti-mouse CD5 – 53-7.3 – CAGCTCAGTGTGTTG
 anti-mouse CD62L – MEL-14 – TGGGCCTAAGTCATC
 anti-mouse CD93 (AA4.1, early B lineage) – AA4.1 – GGTATTTCTGTGGT
 anti-mouse F4/80 – BM8 – TTAACCTCAGCCCGT
 anti-mouse Fc ϵ R1 α – Mar-1 – AGTCACCTCGAAGCT
 anti-mouse I-A/I-E – M5/114.15.2 – GGTCACCAAGTATGAT
 anti-mouse NK-1.1 – PK136 – GTAACATTACTCGTC
 anti-mouse Siglec H – 551 – CCGCACCTACATTAG
 anti-mouse TCR β chain – H57-597 – TCCTATGGGACTCAG
 anti-mouse TCR γ/δ – GL3 – AACCCAAATAGCTGA
 anti-mouse TER-119/Erythroid Cells – TER-119 – GCGCGTTTGTGCTAT
 anti-mouse Ly-6A/E (Sca-1) – D7 – TTCCTTTCCTACGCA
 anti-mouse CD45.2 – 104 – CACCGTCATTCAACC
 anti-mouse CD3 – 17A2 – GTATGTCCGCTCGAT
 anti-mouse CD274 (B7-H1, PD-L1) – MIH6 – TCGATTCCACCAACT
 anti-mouse/rat/human CD27 – LG.3A10 – CAAGGTATGTCACTG
 anti-mouse CD20 – SA275A11 – TCCACTCCCTGTATA
 anti-mouse CD357 (GITR) – DTA-1 – GGCACCTGTAAACAT
 anti-mouse CD137 – 17B5 – TCCCTGTATAGATGA
 anti-mouse CD134 (OX-40) – OX-86 – CTCACCTACCTATGG
 anti-mouse CD69 – H1.2F3 – TTGTATTCCGCCATT
 anti-mouse CD127 (IL-7R α) – A7R34 – GTGTGAGGCACTCTT
 anti-mouse CD86 – GL-1 – CTGGATTGTGTATC
 anti-mouse CD103 – 2E7 – TTCATTAGCCCGCTG
 anti-mouse CD64 (Fc γ R1) – X54-5/7.1 – AGCAATTAACGGGAG
 anti-mouse CD150 (SLAMF) – TC15-12F12.2 – CAACGCCTAGAAACC
 anti-mouse CD24 – M1/69 – TATATCTTGGCCGCA
 anti-human/mouse integrin β 7 – FIB504 – TCCTTGGATGTACCG
 anti-mouse CD106 – 429 (MVCAM.A) – CGTTCCTACCTACCT
 anti-mouse CD8b (Ly-3) – YTS156.7.7 – TTCCTCTATGGAGC
 Rat IgG1, κ isotype Ctrl – RTK2071 – ATCAGATGCCCTCAT
 Rat IgG1, λ isotype Ctrl – G0114F7 – GGGAGCGATTCAACT
 Rat IgG2a, κ isotype Ctrl – RTK2758 – AAGTCAGGTTGTTTT
 Rat IgG2c, κ isotype Ctrl – RTK4174 – TCCAGGCTAGTCATT
 Armenian Hamster IgG Isotype Ctrl – HTK888 – CCTGTCATTAAGACT
 anti-mouse/human KLRG1 (MAFA) – 2F1/KLRG1 – GTAGTAGGCTAGACC
 anti-mouse CD223 (LAG-3) – C9B7W – ATCCGTCCTTAAGG
 anti-mouse CD163 – S15049I – GAGCAAGATTAAGAC
 anti-mouse CD49b – HM α 2 – CGCGTTAGTAGAGTC
 anti-mouse CD172a (SIRP α) – P84 – GATTCCCTTGAGCA
 anti-mouse CD48 – HM48-1 – AGAACCGCCGAGTT
 anti-mouse CD170 (Siglec-F) – S17007L – TCAATCTCCGTCGCT
 anti-mouse CD169/Siglec-1 – 3D6.112 – ATTGACGACAGTCAT
 anti-mouse CD71 – RI7217 – ACCGACCAAGTAGACA
 anti-mouse CD41 – MWReg30 – ACTTGGATGGACACT
 anti-mouse IgM – RMM-1 – AGCTACGCATTCAAT
 anti-mouse CD301a – LOM-8.7 – TGTATTTACTCACCG
 anti-mouse CD304 (Neuropilin-1) – 3E12 – CCAGCTCATTCAACG
 anti-mouse CD36 – HM36 – TTTGCCGCTACGACA
 anti-mouse CD38 – 90 – CGTATCCGTCTCCTA
 anti-mouse CD55 (DAF) – RIKO-3 – ATTGTTGTCAGACCA
 anti-mouse CD63 – NVG-2 – ATCCGACACGTATTA

anti-mouse CD68 – FA-11 – CTTTCTTCACGGGA
 anti-mouse CD79b (IgB) – HM79-12 – TAACTCAGTGCGAGT
 anti-mouse CD83 – Michel-19 – TCTCAGGCTTCCTAG
 anti-mouse CX3CR1 – SA011F11 – CACTCTCAGTCCTAT
 anti-mouse CD301b – URA-1 – CTTGCCTTGCGATTT
 anti-mouse Tim-4 – RMT4-54 – TGCTGGAGGGTATTC
 anti-mouse/rat XCR1 – ZET – TCCATTACCCACGTT
 anti-mouse/rat CD29 – HMβ1-1 – ACGCATTCCCTGTGT
 anti-mouse IgD – 11-26c.2a – TCATATCCGTTGTCC
 anti-mouse CD11a – M17/4 – AGAGTCTCCCTTTAG
 anti-mouse CD200R (OX2R) – OX-110 – ATTCTTCCCTCTGT
 anti-mouse CD200R3 – Ba13 – ATCAACTTGGAGCAG
 anti-mouse CD138 (Syndecan-1) – 281-2 – GCGTTTGTATGTACT
 anti-mouse CD317 (BST2, PDCA-1) – 927 – TGTGGTAGCCCTTGT
 anti-mouse CD9 – MZ3 – TAGCAGTCACTCCTA
 anti-mouse CD371 (CLEC12A) – 5D3/CLEC12A – GCGAGAAATCTGCAT
 anti-mouse CD22 – OX-97 – AGGTCTCTCTGGAT
 anti-mouse IL-33Rα (IL1RL1, ST2) – DIH9 – GCGATGGAGCATGTT
 anti-mouse Ly49H – 3D10 – CCACTAGGCTTATTA
 anti-mouse Ly49D – 4E5 – TATATCCCTCAACGC
 anti-mouse Ly-49A – YE1/48.10.6 – AATTCCGTCAGATGA
 anti-mouse CD185 (CXCR5) – L138D7 – ACGTAGTCACCTAGT
 anti-mouse CD49a – HMα1 – CCATTCATTTGTGGC
 anti-mouse CD1d (CD1.1, Ly-38) – 1B1 – CAACTTGGCCGAATC
 anti-mouse CD226 (DNAM-1) – 10E5 – ACGCAGTATTTCGA
 anti-mouse CD199 (CCR9) – CW-1.2 – CCCTCTGGTATGGTT
 anti-mouse JAML – 4E10 – GTTATGGTTCGTGTT
 anti-mouse CD272 (BTLA) – 6A6 – TGACCCTATTGAGAA
 anti-mouse PIR-A/B – 6C1 – TGTAGAGTCAGACCT
 anti-mouse CD26 (DPP-4) – H194-112 – ATGGCCTGTCATAAT
 anti-mouse CD270 (HVEM) – HMHV-1B18 – GATCCGTGTGCCTA
 anti-mouse CD2 – RM2-5 – TTGCCGTGTGTTAA
 anti-mouse CD120b (TNF R Type II/p75) – TR75-89 – GAAGCTGTATCCGAA
 anti-mouse CD40 – 3/23 – ATTTGTATGCTGGAG
 anti-mouse CD31 – 390 – GCTGTAGTATCATGT
 anti-mouse CD107a (LAMP-1) – 1D4B – AAATCTGTGCCGTAC
 anti-mouse/rat CD61 – 2C9.G2 (HMβ3-1) – TTCTTTACCCGCCTG
 anti-mouse VISTA (PD-1H) – MIH63 – ACATTTCCCTTGCCCT
 anti-mouse CD186 (CXCR6) – SA051D1 – TGTCAGGTTGTATTC
 anti-mouse CD159a (NKG2AB6) – 16A11 – GTGTTTGTGTTCCCTG
 anti-mouse Ly108 – 330-AJ – CGATTCTTTGCGAGT
 anti-mouse CD160 – 7H1 – GCGTATGTCAGTACC
 anti-mouse CD85k (gp49 Receptor) – H1.1 – ATGTCAACTCTGGGA
 anti-mouse CD51 – RMV-7 – GGAGTCAGGGTATTA
 anti-mouse CD94 – 18d3 – CACAGTTGTCCGTGT
 anti-mouse CD205 (DEC-205) – NLDC-145 – CATATTGGCCGTAGT
 anti-mouse CD155 (PVR) – TX56 – TAGCTTGGGATTAAG
 anti-mouse/rat CD81 – Eat-2 – TTGTCACTCACTTCC

Validation

All the antibodies are from commercial sources and have been validated by the vendors and their validation data are available on the manufacturer's websites.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

B16-F10 (ATCC), SK-Mel-2 (Freedman Lab, University of Minnesota), primary epidermal melanocytes (ATCC), Yumm 1.7 (Rothlin Lab, University of Minnesota), A375 (Costalonga Lab, University of Minnesota)

Authentication

None of the cell lines were authenticated.

Mycoplasma contamination

B16-F10 cells were tested for mycoplasma contamination and were negative. All other cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines
(See [ICLAC](#) register)

No commonly misidentified cell lines were used.

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

C57BL/6J (B6) and TCRα KO (B6.129S2-Tcratm1Mom/J, Jax#002116) female mice were purchased from The Jackson Laboratory (Bar

Laboratory animals	Harbor, ME) and were maintained in specific-pathogen free conditions at the University of Minnesota. OT-I transgenic mice crossed to Perforin-1 knockout B6 mice (C57BL/6-Prf1tm1Sdz/J), which were a gift from the Blazar laboratory, were bred and maintained in house. CD45.1+ OT-I mice were fully backcrossed to C57BL/6J mice and maintained in our animal colony. Sample size was chosen on the basis of previous experience. No method of randomization was used during group allocation, and investigators were not blinded. All mice used in experiments were 5-14 weeks of age. All mice were used in accordance with the Institutional Animal Care and Use Committees guidelines at the University of Minnesota.
Wild animals	No wild animals were used.
Reporting on sex	Only female mice were used in all mouse experiments.
Field-collected samples	No field collected samples were used.
Ethics oversight	All mice were used in accordance with the Institutional Animal Care and Use Committees guidelines at the University of Minnesota.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	<i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>
Novel plant genotypes	<i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i>
Authentication	<i>Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.</i>

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Cell isolation for CITE-seq After being excised, B16 tumors were minced on parafilm and digested in RPMI 1640 containing 5% FBS, 2 mM MgCl₂, 2mM CaCl₂ (digestion buffer) and 1mg/mL Collagenase type IV and 0.5mg/mL Dnase I, and incubated at 37°C with constant shaking. After 30 minutes, the remaining tissue pieces were gentleMACS dissociated. Fluorescence-activated cell sorting for CITE-seq Single-cell suspensions of B16 melanoma tumors were prepared from OT-I chimeras as described above. Live cells were enriched using MojoSort Mouse Dead Cell Removal Kit (BioLegend, 480157) and Fc receptors were blocked by incubating cells with anti-mouse CD16/CD32 (2.4G2, 70-0161-M001, Tonbo Biosciences) for 10 minutes on ice. Live-enriched single cell suspensions were stained with Live/Dead dye (Ghost-dye 780, Tonbo Biosciences), fluorescent-conjugated antibodies, oligonucleotide-conjugated Biolegend TotalSeq A antibodies, and TotalSeq A hashtag antibodies. Tissue processing and flow cytometry Inguinal LNs (tumor-draining and non-tumor-draining) were harvested and placed in RPMI 1640 supplemented with 5% FBS. LNs were mechanically punctured with fine-tipped forceps, followed by enzymatic digestion in 4 mg/mL collagenase type IV (C6885, Sigma-Aldrich) for 30 minutes at 37°C. Samples were then passed through a 70 µm cell strainer (Falcon, 352235) to obtain single-cell suspensions. B16 tumors were excised, weighed, minced, and dissociated using gentleMACS C tubes according to the manufacturer's protocol. Tumor suspensions were filtered through a 70 µm strainer (Corning, 352350) prior to downstream analyses. Surface staining was performed for 1 hour at room temperature in DPBS (Hyclone, SH30028.03) containing 2% BSA (A1009, Sigma-Aldrich) and 1% sodium azide. Intracellular staining was conducted using the Transcription Factor Staining Buffer Kit (Tonbo Biosciences, TNB-1213-L150) according to the manufacturer's instructions.
Instrument	BD LSR II and LSR Fortessa and FACS Aria II

Software	BD FACS DIVA was used for collection and analysis was done in FlowJo v10.
Cell population abundance	Not applicable.
Gating strategy	Fluorescence-activated cell sorting for CITE-seq OT-I T cells (Live, CD8a+, and CD45.1+), endogenous polyclonal CD8+ (Live, CD8a+, and CD45.2+), CD4+ (Live, CD4+), NK cells (Live, CD8-, CD4-, and NK1.1+), B cells (Live, CD8-, CD4-, and CD19+MHC-II+), monocytic cells (Live, CD8-, CD4-, NK1.1-, CD19-, and Ly6C+), dendritic cells (Live, CD8-, CD4-, NK1.1-, CD19-, Ly6C-, and CD11c+MHC-II+), melanoma cells (SSC-A-high), and stromal cells (Live, CD45.2-, and CD45.1-) were sorted into PBS (without Ca/Mg) with EDTA and FBS using a FACS Aria II (BD Biosciences). See gating strategy in figure S3. Myeloid cell gating in tumor and LN Tumor-infiltrating monocytes/macrophages were identified as CD11b⁺Ly6C⁺Lineage⁻F4/80⁺ cells, and dendritic cells as Ly6C⁻Lineage⁻MHCII⁺CD11c⁺CD103⁺/⁻ cells. In inguinal lymph nodes, monocytes/macrophages were defined as CD11b⁺Ly6C⁺Lineage⁻F4/80⁺, and dendritic cells as Ly6C⁻Lineage⁻MHCII⁺CD11c⁺CD103⁺/⁻. Migratory dendritic cells were gated as MHCII^{hi}CD11c^{mid/lo}, and resident dendritic cells as MHCII^{mid/lo}CD11c^{hi}. Lineage markers included B220, Ly6G, and Thy1.2 (CD90.2).

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.