Long-distance modulation of bystander tumor cells by CD8⁺ T-cell-secreted IFN-γ

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T-cell-secreted interferon (IFN)-γ can exert pleiotropic effects on tumor cells that include induction of immune checkpoints and antigen presentation machinery components, and inhibition of cell growth. Despite its role as a key effector molecule, little is known about the spatiotemporal spreading of IFN-γ secreted by activated CD8⁺ T cells within the tumor environment. Using multiday intravitral imaging, we demonstrate that T cell recognition of a minor fraction of tumor cells leads to sensing of IFN-γ by a large part of the tumor mass. Furthermore, imaging of tumors in which antigen-positive and antigen-negative tumor cells are separated in space reveals spreading of the IFN-γ response, reaching distances of > 800 μm. Notably, long-range sensing of IFN-γ can modify tumor behavior, as shown by both induction of PD-L1 expression and inhibition of tumor growth. Collectively, these data reveal how, through IFN-γ, CD8⁺ T cells modulate the behavior of remote tumor cells, including antigen-loss variants.

CD8⁺ cytotoxic T lymphocytes (CTLs) play a central role in immune-mediated control of cancer in both preclinical models and cancer patients. First, CD8⁺ T cells that recognize either shared antigens or patient-specific neoantigens are frequently observed in cancer lesions, and the intratumoral presence of CD8⁺ T cells forms a positive prognostic marker in many cancer types. Second, interference with immune checkpoint molecules, such as CTLA-4 and PD-1, which modulate the activity of T cells, has resulted in prolonged clinical benefits in cancer patients. Furthermore, clinical response to PD-1-blocking antibodies has been associated with the density of intratumoral CD8⁺ T cells before treatment, and proliferation of intratumoral CD8⁺ T cells has been observed in responding patients. Third, and most directly, infusion of autologous, tumor-infiltrating, lymphocyte-derived T cell products, including purified CD8⁺ T cell products, has shown an approximately 50% objective response rate in metastatic melanoma and has been demonstrated to induce regression of a subset of epithelial cancers.

The release of perforin- and granzyme-containing cytotoxic granules toward target tumor cells forms a relatively well-understood mechanism of action of CD8⁺ T cells in tumor control. However, it has been called into question whether such direct tumor cell killing can explain tumor control at the low T cell:tumor cell ratios that are observed in tumors. Next to the secretion of perforin and granzymes, CTLs also respond to antigen encounter by the secretion of cytokines, such as IFN-γ and tumor necrosis factor α (TNF-α), which may modify the behavior of responding tumor cells. As an example, signaling through the IFN-γ receptor (IFN-γR) can induce the expression of the CXCL9, CXCL10 and CXCL11 chemokines, which induce migration of activated T cells to the tumor site. In addition, IFN-γR signaling leads to the enhanced expression of immune checkpoint molecules, such as PD-L1 and PD-L2, but also increases the expression of components of the antigen presentation machinery. Finally, IFN-γ can have direct cytotoxic and cytostatic effects on both cancer cells and stromal cells in the tumor mass, inducing cell cycle arrest, cellular senescence or cell death, depending on the (tumor) cell type involved.

Evidence that these downstream effects of IFN-γR signaling are of relevance in T-cell-mediated tumor control comes from several observations. Early work in mice by Schreiber and colleagues demonstrated that tumor cells that lack the IFN-γR can be less sensitive to T-cell-mediated clearance because of their reduced major histocompatibility complex (MHC) expression. More recently, inactivation of components of the IFN-γR pathway, such as STAT1, Jak1, Jak2 and IFN-γR1 and IFN-γ-2, was shown to result in resistance to CD8⁺ T cell attack in a series of in vitro and in vivo CRISPR-based genetic screens. In addition, enhanced sensitivity to CD8⁺ T cell attack was obtained on loss of a chromatin regulator that suppresses sensitivity of tumor cells to IFN-γ. Importantly, inactivating mutations in the IFN-γR signaling pathway have been demonstrated to promote tumor cell outgrowth in human tumors, and such mutations have been identified in tumors that either relapsed after anti-PD-1 treatment or showed upfront resistance to anti-CTLA-4 treatment and anti-PD-1 treatment. Next to the direct effects of IFN-γ on tumor cells themselves, T-cell-derived IFN-γ has also been shown to induce tumor regression in preclinical models by targeting stromal cells, such as endothelial cells in the tumor vasculature.

In spite of this evidence for a widespread role of IFN-γ signaling in tumor control, it is not well understood how different cells in the tumor microenvironment (TME) encounter CD8⁺ T-cell-derived IFN-γ. In particular, a number of studies have provided evidence that transfer of peptide antigens to MHC class I molecules on stromal cells (that is, through peptide cross-presentation) can result in the IFN-γ-mediated killing of these cells. However, in other studies it remains unclear whether IFN-γR signaling may also be induced on ‘bystander cells’ that are not directly recognized by T cells. Such a bystander effect of CD8⁺ T-cell-derived IFN-γ in tumor tissue would be of potential interest, because it would allow activated T cells to exert effects beyond the cells they can directly contact, including tumor cell subclones that have lost T-cell–recognized antigens.

For CD4⁺ helper T cells, it has previously been established that secreted IFN-γ can induce expression of IFN-γ-responsive genes in...
cells outside parasite-infected areas\textsuperscript{11,12}, with responding cells up to 80 μm (that is, approximately four to eight cell layers) away from the closest infected cell\textsuperscript{11}. By the same token, production of IFN-γ and TNF-α by intratumoral CD8+ T cells has been shown to induce senescence in tumor cells that are deficient for MHC class II\textsuperscript{13}. In the case of cytotoxic CD8+ T cells, seemingly contradictory results have been obtained. Specifically, in vitro and in vivo analyses of CD8+ T-cell-target conjugates indicate that the delivery of IFN-γ is directed toward the immunologic synapse that is formed between these cells\textsuperscript{14,15}, suggesting that the IFN-γ signal emitted after T cell receptor (TCR) triggering would primarily reach the involved target cell. In contrast, robust IFN-γR signaling by bystander cells that lacked a specific T cell antigen has been observed in in vitro astrocyte–T cell cocultures, an observation that has been explained by synaptic leakage of CD8+ T-cell-produced IFN-γ\textsuperscript{16}. At present, in vivo data on the potential spread of CD8+ T-cell-derived IFN-γ, including its spatiotemporal behavior, are lacking and, in the present study, we set out to analyze: (1) whether antigen-negative cells can sense the IFN-γ that is secreted on antigen encounter by tumor-specific CD8+ T cells; (2) how such a signal spreads through the tumor mass in space and time; and (3) whether the long-range sensing of IFN-γ can yield a functional response of bystander tumor cells that cannot be directly recognized by T cells. The data obtained demonstrate that tumor recognition by even a limited number of intratumoral CD8+ T cells induces a gradient of IFN-γ that reaches tumor cells that have been removed by many cell layers. The observed long-range sensing of CD8+ T-cell-derived IFN-γ has implications for both preemptive tumor cell resistance to immune attack and the control of antigen-loss variants that arise during clonal evolution.

Results

To analyze the spatial spreading of CD8+ T-cell-derived IFN-γ in vivo, we aimed to set up a system in which the activity of IFN-γ-induced signaling in tumor cells could be followed in both space and time. To this end, we first created an IFN-γ-sensing (IGS) reporter that induces the expression of the Katushka fluorescent protein in cells after IFN-γR triggering, and that consists of an IFN-γR-signaling responsive promoter containing a series of gamma interferon activation sites (GAS elements), followed by the Katushka fluorescent protein sequence (Fig. 1a). To validate this IGS reporter, human ovarian carcinoma (OVCAR5) cells modified with the reporter were exposed to either IFN-γ or IFN-α. In agreement with expectations, IGS reporter-modified tumor cells showed robust Katushka expression within 24 h of exposure to low concentrations of IFN-γ (half-maximal concentration of approximately 3 ng ml\textsuperscript{-1}), with a maximal Katushka signal 48 h after stimulation (Fig. 1b and Extended data Fig. 1a). In contrast, exposure to IFN-α resulted in only low-level Katushka expression when used at high concentrations (10,000 U ml\textsuperscript{-1}; Extended data Fig. 1b). Comparable reporter expression kinetics were observed in MDA-MB-231 breast cancer cells (Extended data Fig. 2a), and induction of reporter expression was highly correlated with induction of PD-L1, a known endogenous IFN-γ-inducible protein (Fig. 1c and Extended data Fig. 2b). Notably, prolonged IFN-γ exposure of OVCAR5 cells induced cell death (Extended data Fig. 1c,d), thereby providing a second, late and functional measure of IFN-γ sensing.

To measure in vivo IFN-γ sensing by bystander cells that cannot be directly recognized by CD8+ T cells, we generated chimeric tumors that contain small adjacent groups of tumor cells that do and do not form T cell targets, by injection of mixtures of neoantigen-positive and neoantigen-negative tumor cells (Fig. 2a). Analysis of expression of the IGS reporter in the latter cells provides a means to determine whether tumor cell subclones that lack antigen are influenced by the IFN-γ that is secreted by activated T cells in close proximity. Previous data have shown that, although bystander cells can respond to IFN-γ released by CD8+ T cells in in vitro assays\textsuperscript{17,18}, the IFN-γ-containing vesicles in CD8+ T cells are selectively transported toward the immunologic synapse\textsuperscript{19,20}, suggesting localized effector activity to the target cell. To address whether the IFN-γ that is secreted by activated intratumoral CD8+ T cells in vivo is sensed by bystander tumor cells, a GFP+ (green fluorescent protein-positive) OVCAR5 tumor cell line was generated that expresses the patient-derived mutant cyclin-dependent kinase 4 (CDK4\textsubscript{mut}) neoantigen\textsuperscript{32}. Subsequently, mixtures of these neoantigen-positive (Ag+) tumor cells, together with GFP+ IGS reporter cells that lack the mutant CDK4 neoantigen (Ag−), were injected into NOD-scid IL2rg\textsuperscript{null}β2m\textsuperscript{null} (NSG-β2m-\textsuperscript{−/−}) mice. In the absence of tumor-specific T cells, mice developed chimeric tumors that were composed of small, intermingled groups of both tumor cell populations (Extended data Fig. 3a). To analyze to what extent bystander tumor cells that lack a T-cell-recognized antigen respond to CD8+ T-cell-secreted IFN-γ, mice were treated with CDK4\textsubscript{mut}-specific, TCR-transduced, CD8+ T cells. Remarkably, although only a small fraction of bystander tumor cells showed detectable Katushka expression in control mice (3.2±2.0%), a large fraction of bystander tumor cells (64.0±8.3%) demonstrated pronounced Katushka expression in tumors with an ongoing tumor-specific T cell response (Fig. 2b,c and Extended data Fig. 4a), an observation that was replicated in the MDA-MB-231 tumor model (Extended data Fig. 2c). This reporter expression by bystander tumor cells required the presence of both tumor-specific CD8+ T cells and a subpopulation of antigen-positive tumor cells (Fig. 2d), and was observed even when the antigen-positive tumor cell subclone formed only a minor fraction (10%) of the tumor cell mass (Fig. 2d). To assess whether the observed reporter

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**Fig. 1 | Characterization of the IGS reporter system.** a, Schematic representation of the IGS reporter system. b, Percentage Katushka-expressing cells of IGS reporter-modified GFP+ OVCAR5 cells on incubation with the indicated concentrations of recombinant IFN-γ. Bar graph shows the mean of n = 3 technical replicates and are representative data of two independent experiments. c, Median fluorescence intensity (MFI) of PD-L1 staining as a function of median Katushka fluorescence intensity of GFP+ Ag+ IGS reporter cells incubated for 24 h with recombinant IFN-γ under the indicated conditions. Representative data of two independent experiments are depicted.
expression by Ag− cells was directly due to IFN-γ sensing, or could be explained by, for example, type I IFN signaling36, we generated tumors in which Ag− IGS reporter cells that lacked the IFN-γR were intermingled with low numbers of Ag+ tumor cells. In this setting, no Katushka reporter signal was observed in recipients of tumor-tumor cell mixtures (10%) and CFP− IGS reporter cells (90%) (left two bars), or solely consisting of CFP+ IGS reporter cells (right two bars) were generated as in b. Mice were then treated with either HBSS (control) or 5 × 10^6 CDK4r-proficient CFP+ IGS reporter cells and tumors were harvested at day 3 after treatment. Bar graphs depict mean percentage ± s.d. of Katushka+ reporter cells with n = 3 mice per group. Representative data of two biologically independent experiments are depicted. Fold differences (FD) were calculated by dividing the means of T-cell-treated and HBSS-treated groups. e. Mouse tumors consisting of 10–90% mixtures of GFP−Ag+ tumor cells and IFN-γR-deficient or IFN-γR-proficient CFP+ IGS reporter cells were generated as in b. Mice were then treated with 5 × 10^6 CDK4r-proficient CFP+ IGS tumor cells, and tumors were harvested at day 3 after treatment. Bar graphs depict mean percentage ± s.d. of Katushka+ reporter cells (n = 5 mice per group). Representative data of two independent experiments are depicted (**P = 0.0079). Two-tailed Mann–Whitney U-tests were performed for all statistical analyses.

The observed increase of Katushka+ reporter cells at later time-points could be explained both by an increased number of local regions of T cell activity and by the gradual spreading of an IFN-γ signal through the tumor mass. To understand whether the activity of IFN-γ is progressively observed at larger distances from sites of antigen presentation, we measured the distance of each Katushka+ bystander cell to the nearest antigen-presenting tumor cell at different times post-T cell transfer. Importantly, as antigen-positive tumor cells are expected to be cleared over time due to T-cell-mediated killing, it was essential to focus this analysis on timepoints at which the average distance between antigen-positive tumor cells and all bystander tumor cells (that is, irrespective of their Katushka expression) is constant. To determine this, we first measured the total volume of GFP−Ag+ cells in tumors over time, observing a substantial loss of GFP+ tumor area starting around 48 h after T cell infusion (Extended data Fig. 5a). In line with this, the median distance between the entire pool of bystander tumor cells, irrespective of reporter expression, and antigen-positive tumor cells was stable during the first 40 h after T cell injection (Extended data Fig. 5b), providing a window to measure the spread of the IFN-γ response up to that time. Notably, analysis of the distances between Katushka+ bystander cells and the nearest antigen-expressing tumor cell within this time window revealed a pronounced increase over time that was reproducibly observed in different mice (Fig. 4a), with reporter-positive
Fig. 3 | Kinetics of IFN-γ sensing by bystander tumor cells. 

**a.** Illustration of imaging window setup and experimental timeline. At the indicated timepoints in the 0 h to 120 h time window, intravital imaging of tumor lesions was performed.

**b.** Sequential, intravital, two-photon imaging of tumors consisting of 10% GFP+Ag+ tumor cells (green) and 90% CFP+IGS reporter bystander tumor cells (cyan) was initiated 14 d after injection of tumor cells (8×10⁶ cells total) into the mouse mammary gland. On IGS reporter activation, cells gain Katushka signal (white in upper panel, red in lower panel). A representative imaging series of a tumor region with Katushka reporter expression (top panel) and merger of GFP+Ag+ (green), CFP+Ag− (cyan) and Katushka reporter (red) (lower panel) signal is depicted over time. Data are representative of **c.** 

**c.** Quantification of the percentage of IGS Katushka+ reporter cells in the Ag− cell population showing the mean ± s.d. over time. Symbols represent data from different animals (n = 5 mice for t = 0, 16, 24 and 32 h; n = 4 mice for t = 40, 48 and 72 h; and n = 3 mice for t = 120 h, obtained in all independent experiments).

IVM, intravital microscopy.
cells located up to 100–300 μm away from the nearest Ag⁺ cell at the latest timepoint analyzed (representative graph, Fig. 4b,c).

Although the above data directly demonstrated how the IFN-γ response in tumors can spread from sites of T cell activation (that is, areas that harbor Ag⁺ tumor cells), accurate long-range measurements of IFN-γ sensing were precluded by the fact that the large majority of bystander cells are located within 100 μm of the nearest Ag⁺ tumor cell (Extended data Fig. 3), providing few data...
to quantify spreading at greater distances. To be able to measure long-distance sensing of CD8+ T-cell-derived IFN-γ, we generated tumors that consist of large islands of Ag+ cells and Ag− IGS reporter cells (Fig. 4d), using Matrigel-embedded Ag− IGS reporter cell spheres, which were then injected with a low number of Ag+ cells (Methods). After tumor outgrowth and antigen-specific T cell transfer, the resulting tumors showed strictly defined Ag+ and Ag− areas, with antigen-reactive CD8+ T cells locating preferentially to the Ag+ areas of these tumors (representative tumor in Fig. 4e and Extended data Fig. 6a,b). This tumor setup resembles the islands of distinct tumor cell populations seen in human tumors as read by β2m expression (representative images in Extended data Fig. 7), with increased CD8+ T cell counts in the β2m+ areas (Extended data Fig. 7; discrete β2m+ and β2m− areas observed in 16/51 tumors analyzed). Strikingly, analysis of the Katushka reporter signal in the Ag+ tumor lobe after T cell transfer revealed a pronounced IFN-γ response that started at the interface between the Ag+ and Ag− tumor lobes and extended more than 800 μm into bystander tumor territory (n = 7 mice). In addition, whereas the CFP signal (present in all Ag+ IGS reporter cells, irrespective of reporter activation) was constant over distance, analysis of consecutive rings of bystander tumor tissue showed a gradual waning of Katushka reporter signal as a function of distance to the Ag+ tumor compartment (Fig. 4e,f).

Based on the observation that T cell density is higher in the Ag+ tumor area than in the Ag− area, and the observation that T cells cease to produce IFN-γ within hours of TCR signaling termination (with a reduction of more than 80% in the first 2 h; Extended data Fig. 6c,d), we interpret the observed gradient in Katushka signal to occur as a consequence of IFN-γ diffusion. However, reporter activation by bystander cells that are approached by tumor-specific T cells that have left the Ag+ area and continue to secrete IFN-γ remains a formal possibility. Most importantly, these data indicate that CD8+ T cell activation can induce activity of the IFN-γR signaling pathway in cells that are located more than 30–40 cell layers away from the site of antigen−T cell interaction.

Long-range sensing of IFN-γ may modulate the behavior of tumor cells in a negative way by inducing a ‘preemptive’ expression of immune checkpoints. Alternatively, sensing of T-cell-secreted IFN-γ by bystander tumor cells may potentially also boost tumor control by inducing enhanced MHC expression or the expression of chemokines such as CXCL9 and CXCL10, or by directly inducing tumor cell death. To explore the occurrence of preemptive immune checkpoint expression, we generated tumors in which Ag+ bystander cells were intermingled with low numbers of Ag− tumor cells, and measured the PD-L1 expression after T cell transfer. Importantly, after T cell treatment, a large fraction (71 ± 12%) of bystander tumor cells demonstrated a prominent induction of PD-L1 expression (Fig. 5a and Extended data Fig. 2d). In a second test for the functional consequences of long-range IFN-γ sensing, we exploited the prior observation that OVCAR5 cells undergo cell death on prolonged IFN-γ exposure (Extended data Fig. 1c,d). In line with this, after in vitro coculture with a combination of Ag+ tumor cells and antigen-specific T cells, Ag− bystander tumor cells that were IFN-γR proficient were selectively lost relative to Ag− bystander cells that lacked the IFN-γR (Fig. 5b and Extended data Fig. 8). Subsequently, we assessed whether IFN-γ sensing can also form a selective disadvantage of bystander tumor cells in vivo. For this purpose, mice were inoculated with tumor cell mixtures that consist of Ag+ tumor cells, and two separate bystander tumor cell populations that were either IFN-γR proficient or IFN-γR deficient. After tumor cell outgrowth, mice were either mock treated or received tumor-specific T cells, and the relative numbers of the antigen-expressing tumor cells and the two bystander tumor cell populations were compared. In agreement with expectations, treatment of mice with CDK4R+/−specific CD8+ T cells resulted in a major decrease in CDK4R+/− neoantigen-positive tumor cells. Notably, although roughly equal numbers of IFN-γR-proficient and IFN-γR-deficient tumor cells were recovered in control-treated mice, an almost full depletion of IFN-γR-proficient bystander tumor cells was observed in tumors with an ongoing tumor-specific T cell response (Fig. 5c).

In additional experiments, tumors that contained IFN-γR-proficient bystander cells were shown to display an impaired tumor growth after T cell treatment, compared with tumors that contained IFN-γR-deficient bystander cells (Fig. 5d). Finally, direct analysis of the outgrowth of...
the bystander tumor cell compartment by bioluminescence demonstrated impaired outgrowth of IFN-γR-proficient bystander cells, relative to IFN-γR-deficient bystander cells in the same animal, in T-cell-treated mice but not in control mice (Fig. 5e). To assess whether the observed deletion of Ag− tumor cells through IFN-γR signaling could be due to antigen cross-presentation, we generated IFN-γR-proficient and IFN-γR-deficient bystander cells lacking the HLA-A*02 heavy chain that forms the restriction element for the CDK4R neoantigen. Also, in a setting in which bystander tumor cells lack both antigen and the relevant human leukocyte antigen (HLA) class I allele, widespread IFN-γR-dependent deletion of bystander tumor cells was observed after a T cell response had been initiated (Fig. 5f). Impaired outgrowth of bystander tumor cells was observed in a setting in which Ag+ tumor cells and bystander cells were either intermingled or separated into larger domains, but was more prominent in the former case (Fig. 5g). This suggests that IFN-γ is most likely to exert a selective pressure on the bystander tumor cell compartment in an early phase of genetic diversification of tumor tissues, when tumor cell compartments that can and cannot be recognized by T cells are still in close proximity.

Discussion
Exposure to IFN-γ can influence cell behavior in a number of ways and can, for instance, inhibit tumor cell growth through induction of apoptosis37, senescence15 and ferroptosis38. The current data provide evidence that CD8+ T cell activity in tumors can modulate the behavior of a large fraction of the tumor mass, even when only a small percentage of the tumor cells in that lesion can be recognized by CD8+ T cells, and even when tumor-antigen-specific T cells are present at low frequencies (T cell:Ag+ target ratio of approximately 1:200; Extended data Fig. 6). Furthermore, through the use of tumors that contain large substructures of Ag+ and Ag− tumor cells, we document that IFN-γ sensing can occur at long distances (>800 μm, equivalent to 30–40 cell layers) from sites of antigen encounter, a substantially larger distance than, for instance, the maximal spread of oxygen derived from capillary blood vessels in tumor tissue (4–8 cell layers)39. The most probable explanation for this widespread IFN-γ sensing would be substantial diffusion of IFN-γ away from the immunologic synapse after T cell activation, but a contribution of T cells that continue to secrete IFN-γ into the surrounding space after disengagement from the antigen-expressing
tumor cells remains a possibility. Of note, exposure of OVCAR5 tumor cells to IFN-γ does not induce an increase in Ifng transcript levels (data not shown), ruling out a feed-forward loop as an underlying mechanism for the observed signal spreading.

With respect to the functional consequences of the observed IFN-γ sensing, we would like to highlight three things. First, the current data strongly indicate that preemptive resistance of tumor cells through IFN-γ-driven expression of inhibitory molecules such as PD-L1, TNFRSF14 and Galectin-9 (ref. 39) can occur well before the actual arrival of CD8+ T cells in a tumor substructure. Second, for those tumors that are sensitive to IFN-γ-mediated killing, growth arrest or senescence, the observed bystander IFN-γ sensing may be considered a fail-safe mechanism in case of antigen loss. In other words, tumor cells that have lost relevant antigens, and are therefore invisible to T cells, may still be cleared by means of 'back-up' cytokine secretion by tumor-specific T cells. Third, besides acting on tumor cells, the widespread effects of IFN-γ can be expected to influence other cell types present in the TME, such as the T cells themselves, potentially inducing apoptosis of activated tumor-antigen-specific T cell clones that express high levels of the IFN-γR1. 40

In conclusion, the ultimate outcome of the observed long-range IFN-γ sensing will be determined by factors such as the antigen presentation potential, expression of inhibitory molecules and immune infiltrate of individual tumors, and inhibition of this sensing could be attractive in certain settings. From a more conceptual point of view, with our increasing understanding of the cytokine and chemokine output of tumor-antigen-specific CD8+ T cells 41-44, it will be of interest to understand the relative spread of molecules such as IFN-γ, Tnf-α and CXCL13, but also to understand how the joint sensing of these molecules collectively modulates the TME on spontaneous or therapy-induced T cell activation.

Methods

IGS reporter generation and viral vectors. To generate the IGS reporter vector, four tandem repeats of the GAS flank by enhancer elements (5' -agtttcatattacattaaatc-3', GAS consensus sequences underlined 45, enhancer elements derived from GAS cis-reporting systems (Aglent, catalog no. 19093)) were cloned in front of the coding sequences of the Katushka fluorescent protein (TurboF635, Evrogen), a P2A element and the CreER2 protein (CreER2 expression was not used in the present study), into a variant of the lentiviral pCDH-puroycin vector (Addgene, catalog no. 2082) in which the CMV promotor had been removed. To generate the CDK4R γ-GFP vector, a sequence encoding the patient-derived, mutant cyclin-dependent kinase epitope (CDK4K4,2- APLFHERGHV), followed by an internal ribosome entry site (IRES) and GFP sequence was inserted into the pMX-retroviral vector (Addgene, catalog no. 3674). To generate the CFP expressing vector, the CFP sequence was inserted into the pMX-retroviral vector. To generate the pCDH-Katushka vector, the Katushka sequence was inserted into the lentiviral pCDH-EF1α vector (Addgene, catalog no. 72266).

For bioluminescence experiments, the lentiviral pLKO-1-Ubc-fire-fly-luciferase-blast was used. As TCR expression vectors, retroviral pMP71 vectors encoding the CDK4R γ-luciferase-blast was used. For TCR expression vectors, retroviral pMP71 vectors (see 'IGS reporter generation and viral vectors'). After transduction, indicated cell populations were sorted on a FACSaria Fusion (BD Biosciences) to >90% purity. Ag+ CFP+ IGS reporter cells were generated by lentiviral transduction of Ag+ CFP+ tumor cells with IGS reporter virus (see 'IGS reporter generation and viral vectors'), followed by selection of the transduced cells in the presence of 2 μg/ml-1 of puromycin (Sigma). Ag+ CFP+Luc+ and Ag+ CFP+IFN-γR1−/−Luc+ cells were generated by lentiviral transduction of Ag+ CFP+ tumor cells with CFP-expressing virus with the pLKO-luciferase vector (see 'IGS reporter generation and viral vectors').

Generation of knockout cell lines. Ag+ CFP+IFN-γR1−/− cells, Ag+ CFP+HLA-A−/− cells and Ag+ CFP+IFN-γR1−/−HLA-A−/− cells were generated using the CRISPR-Cas9 system, by transduction of cells with a plentiCRISPR v2 vector (Addgene, catalog no. 52961) encoding the single guide RNA sequence ACATGAACCTATCGCTATAT (IFN-γR1) or GCCACGTACAGACTGACAGG (HLA-A) using X-tremeGENE (Roche), according to the manufacturer's protocol. Then 48 h after transfection, cells were selected with 2 μg/ml-1 of puromycin (Sigma). For IGS reporter generation, indicated cell populations were sorted on a FACSaria Fusion (BD Biosciences) to >90% purity. Ag+ CFP+IFN-γR1−/−IGS reporter cells were generated by transduction of Ag+ CFP+IFN-γR1−/− cells with IGS reporter virus (see 'IGS reporter generation and viral vectors'). To distinguish between IFN-γR1-proficient and IFN-γR1-deficient cells, IFN-γR1-deficient tumor cells were subsequently transduced with the pCDH-Katushka vector and isolated by cell sorting.

T cell culture and T cell transductions. Retroviral transduction of T cells was performed as described previously. 46 In brief, FLRYR1B8 packaging cells were plated on to six-well plates at 0.5 × 10^6 cells per well. After 24 h, cells were transfected with 3 μg pMP71-CDK4R γ, TCR, pMP71-CDK4R γ, TCR-mOrange2 or pMP71-1D3 TCR (see 'IGS reporter generation and viral vectors'), using X-tremeGENE (Roche), according to the manufacturer's protocol. CD8+ T cells were isolated from peripheral blood mononuclear cells of healthy donors (Sanquin) by using the CD8+ T Cell Isolation Kit (Miltenyi Biotec). Isolated CD8+ T cells were seeded in 24-well plates at a density of 1 × 10^6 cells per well, and stimulated with CD3/CD28 Dynabeads (Life Technologies) in a 1:1 mix of AIm 5 (Gibco) and RPM1 medium (Gibco), supplemented with 10% AB serum (Life Technologies). 100 μl-1 of penicillin (Roche), 100 μg/ml-1 of streptomycin (Roche), 60 μM-1 of interleukin (IL)-2 (Proleukin, Novartis) and 10 ng/ml-1 of IL-15 (Peprotech). After 48 h, virus-containing supernatants were collected and centrifuged to remove cellular debris. Supernatants were subsequently transferred to Retronectin (Takara)-coated, 24-well plates, and plates were centrifuged at 430g for 90 min at 4°C. After centrifugation, virus supernatants were removed and replaced with 5 × 10^5 activated CD8+ T cells per well in medium supplemented with 60 μl-1 of IL-2 and 10 ng/ml-1 of IL-15. After 72 h, cells were stained with an antibody specific for the activated CD8+ T cell constant (see 'Flow cytometry'), and transduction efficiency was determined by flow cytometry. Transduced CD8+ T cells were grown for 3 weeks, receiving fresh medium and cytokines every 3–4 d until used in functional assays or cryopreservation. After cryopreservation, transduced CD8 T cells were thawed and rested for 1 d in 50:50 medium supplemented with 60 μM-1 of IL-2 at 37°C/5% CO2 before experimental use.

In vitro characterization of IGS reporter cells and T-cell-mediated tumor cell killing. Ag+ CFP+ IGS reporter tumor cells proficient or deficient for the IFN-γR were plated at 20,000 cells per well in 48-well plates and received the indicated concentrations of either human IFN-α (Thermo Fisher Scientific) or human IFN-γ (Invirogen). At the indicated timepoints, cells were harvested, stained with IRDye (Invirogen) and analyzed by flow cytometry. To measure T-cell-mediated death of bystander tumor cells, a mixture of GFPAg+, CFPAg+ IFN-γR-proficient and CFPAg+ IFN-γR-deficient OVCAR5 cells was plated at 100,000 cells per well in 6-well plates at a 2:1 ratio, and cells were then incubated with either human balanced salt solution (HBSS; Gibco; or CDK4R γ−specific CD4+ T cells in HBSS at a 1:5 ratio of T cell:total tumor cell). Cell death and total cell counts were analyzed and reported, indicated cell population on day 3 after treatment with IRDye staining and subsequent flow cytometry using AccuCountBlank 15.2 μm beads (Spherotech).

LCK inhibitor experiments. Isolated CD8+ T cells from peripheral blood mononuclear cells (see 'T cell culture and T cell transductions') were activated in 96-well plates coated with 5 μg/ml-1 of plate-bound Ultra-LEAF anti-CD3 antibody (OKT3, Biologend) and 2 μg/ml-1 of plate-bound Ultra-LEAF anti-CD28 antibody (CD28.2, Biologend) at a density of 1 × 10^6 cells per well, in a 1:1 mix of Aim 5 (Gibco) and RPM1 medium (Gibco), supplemented with 10% AB serum (Life
injection of either 200 surgically inserted into the skin of female mice under aseptic conditions, on top Mixed tumors for intravital imaging.

Mice. NOD-scid Il2rg−/−82mAvi mice were obtained from Jackson Laboratories. All animal experiments were approved by the Animal Welfare Committee of the Netherlands Cancer Institute (NKI), in accordance with national guidelines. All animals were maintained in the animal department of the NKI, housed in individually ventilated cage systems under specific-pathogen-free conditions, and received food and water freely. Mice were used at 8–26 weeks of age.

In vivo tumor experiments. Mixed tumors. OVCAR3 cells, 8 × 10⁶, or MDA-MB-231 cells, 5 × 10⁶, were injected subcutaneously into the flank of NOD-SCID +/− mice in 50 μL HBSS and 50 μL Matrigel (Corning), using the indicated mixtures of tumor cell variants.

Segmented tumors. First the major contributing cell population was injected subcutaneously into the flank of NOD-SCID +/− mice in 40 μL HBSS plus 40 μL Matrigel. Subsequently, mice were placed on a 37 °C heating pad for 15 min, while being kept in a 2% CO₂/98% O₂ mixture of 100% humidity. After surgery, mice were treated with buprenorphine (0.01 mg kg⁻¹) and 100 U ml⁻¹ of d-luciferin (Promega) and, after a 5-min incubation while intravenous injection of either 200 μL HBSS or 5 × 10⁶ tumor-specific mOrange2-CDK4ΔE, TCR-transduced or control D3 TCR-transduced CD8⁺ T cells in 200 μL HBSS. On days 0, 1, and 2, mice received injections of 7.2 × 10⁶ IU IL-2 dissolved in 200 μL HBSS, twice daily, with an interval of 6–12 h between injections. At the indicated times after T cell transfer, tumors were intravitaly imaged (see ‘Multiday intravital imaging’), or mice were sacrificed and tumors harvested. Harvested tumors were either injected (see ‘Imaging and analysis of segmented tumors’) or manually minced and enzymatically digested in RPMI medium (Gibco) supplemented with 200 μM LA of collagenase type IV (Gibco) and 200 μg ml⁻¹ of DNase I (Sigma) at 37 °C for 30 min under continuous shaking. Subsequently, cell suspensions were digested through a 70-μm strainer (Falcon) and single-cell suspensions were stained with IR-Dye and anti-CD8 antibody, and analyzed by flow cytometry.

Tumor volume measurement and bioluminescence imaging. Kinetics of tumor growth were analyzed twice a week by caliper measurements. Bioluminescence imaging was performed immediately before treatment and twice per week thereafter. To perform bioluminescence imaging, mice were injected with single-cell suspensions were stained with IR-Dye and anti-CD8 antibody, and analyzed by flow cytometry. For each imaging session, mice were sedated using 0.01 mg kg⁻¹ buprenorphine and 100 U ml⁻¹ of d-luciferin and, after a 5-min incubation while assessed by staining of cells with fluorochrome-labeled antibodies in FACS buffer (0.5% w/v bovine serum albumin (Fisher Scientific) in phosphate-buffered saline) for 20–30 min at 4°C, while protected from light. After incubation, cells were washed twice with FACS buffer before resuspension in FACS buffer for analysis. IR-Dye was used to allow for live cell selection. Multiday intravital imaging. For each imaging session, mice were sedated using isoflurane inhalational anesthesia (~1% isoflurane/compressed air mixture) and received 200 μL sterile phosphate-buffered saline by subcutaneous injection. Mice were then placed in a customized imaging box on the microscope while being kept under constant anesthesia, with the imaging box and the microscope adjusted to 34.5 °C using a climate chamber. After each imaging session, mice were kept at 37 °C until they had fully recovered from anesthesia. Intravital images were acquired using an inverted Leica SP8 Dive system with a Matiai EHP DeepSee laser (Spectra-Physics) and InSight X3 (Spectra-Physics). Three-dimensional (3D) bioluminescence signal that was present in each channel was excluded from the analysis for each signal, the total volume (as a measure of tumor volume) was determined. In addition, for each Ag⁺ cell and Katushka Ag⁺ cell, the distance to the nearest Ag⁺ neighbor was calculated using the distance transformation and intensity center functions.

Imaging and analysis of segmented tumors. Tumors were isolated from mice and placed between two coverslips. From these freshly isolated tumors, 3D slice scans were acquired by two-photon confocal microscopy (same settings as described in multiday intravital microscopy). Using a custom-made ImageJ v1.52i macro (available on request), the mean intensity of CFP and Katushka signal was measured as a function of the distance to the nearest Ag⁺ area. In brief, binary images were created from the GFP (that is, Ag⁺) and CFP (that is, Ag⁻) channels based on a threshold. The binary image from the GFP channel was dilated with steps of 200 μm to create bins of ROIs at increasing distances from Ag⁺ areas. Next, the ROIs at various distances were combined with the binary image of the CFP channel and an AND function. For those ROIs at different distances from the Ag⁺ area with an area larger than 4 × 10⁶ μm², the mean intensity of CFP and Katushka signal in the original image was determined, and the obtained volumes were corrected for background signal as measured in the GFP area. To be able to compare data between experiments, the mean signal intensities per ROI were normalized to the mean intensity measured in the ROI at a distance of 200–400 μm from the Ag⁺ area. Analysis was done on multiple Z-slices per mouse, and mean signal intensity per distance bin was averaged per mouse.

T cell quantification. The number of mOrange2⁻ T cells was quantified in 3D slice scans (500 × 500 × 100 μm³) in the Ag⁺ and Ag⁻ areas of segmented tumors by manual counting using ImageJ v1.52i. The number of tumor cells was estimated by dividing tumor volume by the volume of a spherical tumor cell. The average volume of spherical tumor cells was determined by measuring the diameter of 50 cancer cells, yielding a value of 24 μm³.

Human tumor immunohistochemistry. Human tumor tissue was obtained from 51 patients (no data on specific patient characteristics) in accordance with national guidelines, following opt-out procedure and after approval by the local medical ethical committee (Institutional review board of the NKI). Anton van Leeuwenhoek Hospital). Tumor tissue was collected from surgical specimens after macroscopic examination of the tissue by a pathologist. For each specimen, a fragment was formalin fixed and paraffin embedded or histology. Then, 3-μm sections were cut from formalin-fixed, paraffin-embedded tumor material and slides were stained with anti-CD2 (polyclonal, Dako/Agilent) or anti-CD8 (clone C8/144B, Dako/Agilent) antibodies. Slides were counterstained with Hematoxylin and Blueing Reagent (Ventana Medical Systems). All slides were scanned on the Aperio ScanScope, uploaded on Slide Score (https://www.slidescore.com) and manually assessed for j2m and CD8 expression.

Statistics and reproducibility. All repeated independent experiments showed similar results. Statistical analyses were performed in Prism (GraphPad). Two-tailed Mann–Whitney U-tests and a two-tailed, unpaired t-test with normality test were used. For all boxplots, center-line represents the median, box limits represent upper and lower quartiles, and whiskers represent minimum and maximum values. No statistical analysis was used to predetermine sample size. In the analysis of Fig. 4f, areas smaller than 4 × 10⁴ μm² were excluded from analysis due to the very small numbers of cells present, and therefore high variability in the measurements. No further data were excluded from the analyses. Treatment of tumor-bearing mice (controls/T cells) was randomized. Intravital microscopy analysis was performed blinded, such that the person performing the analysis
did not know the timepoints corresponding to the data. Tumor analysis by flow cytometry was blinded with the person performing the analysis not knowing the treatments given.

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

**Data availability**

Statistical source data for all figures and extended data figures including all independent repeats are provided online.

All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Online content**

Any methods, additional references, Nature Research 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/s43018-020-0036-4.

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Author contributions
M.E.H., L.B., D.P. and I.N.P. performed experiments and analyzed data. L.B. performed multiphoton imaging, F.E.D. and M.E.H. designed and tested the IGS reporter. L.B. and M.E.H. designed imaging analyses. M.E.H. and M.T. performed LCK inhibitor experiments. D.S.T. provided and analyzed human tumor samples. M.E.H., F.E.D., J.v.R. and T.N.M.S. contributed to experimental design. M.E.H., L.B., J.v.R. and T.N.M.S. prepared the manuscript with input from all the coauthors.

Competing interests
The authors declare no competing interests.

Additional information
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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | In vitro IGS reporter cell characteristics. a, Median Katushka fluorescence intensity of IGS reporter-modified CFP+ OVCAR5 cells upon incubation with recombinant IFNγ under the indicated conditions. Bar graph shows mean of n=3 technical replicates, representative data of two independent experiments are depicted. b, CFP+ IGS reporter modified OVCAR5 cells proficient or deficient for the IFNyR were incubated for 48h with the indicated concentrations of recombinant IFNy or IFNα and Katushka expression was analyzed by flow cytometry. Bar graphs show mean of technical duplicates data obtained from one experiment. c, Percentage IR-Dye positive IGS reporter-modified CFP+ OVCAR5 cells upon incubation with recombinant IFNy under the indicated conditions. Bar graph shows mean of n=3 technical replicates. Representative data of two independent experiments are depicted. d, Percentage of IR-Dye positive Ag−CFP+ or Ag−IFNγR−/− OVCAR5 cells after 72h incubation with 100 ng/mL IFNγ. Bar graph shows mean of n=3 technical replicates. Representative data of three independent experiments are depicted.
Extended Data Fig. 2 | IFNγ-induced IGS reporter and PD-L1 expression in MDA-MB-231 cells. **a**, Median Katushka fluorescence intensity of IGS reporter-modified CFP⁺ MDA-MB-231 cells upon incubation with recombinant IFNγ under the indicated conditions. Bar graph shows mean of n=3 technical replicates. Representative data from two independent experiments are depicted. **b**, Median fluorescence intensity of PD-L1 staining as a function of median Katushka fluorescence intensity of CFP⁺Ag⁺ IGS MDA-MB-231 reporter cells incubated for 24h with recombinant IFNγ under the indicated conditions. Plot depicts representative data three technical replicates of two independent experiments. **c**, 20% GFP⁺ cells and 80% CFP⁺ IGS reporter bystander MDA-MB-231 tumor cells (5 x 10⁶ total) were subcutaneously injected in NSG-β2m⁻/⁻ mice. Mice were treated with HBSS (control), 5 x 10⁶ control CD8⁺ T cells or with 5 x 10⁶ CDK4r> L-specific CD8⁺ T cells, and tumors were harvested at day 3 after treatment. Bar graphs depicting mean percentage plus SD of Katushka⁺ reporter cells in control and tumor-specific T cell treated mice, n=5 mice per group. Representative data of two independent experiments are depicted. Two tailed Mann-Whitney U test was performed, with: p=0.3095 (ns); p=0.0317 (*); p=0.0079 (**). **d**, Percentage of PD-L1-expressing cells of IGS reporter-modified CFP⁺ MDA-MB-231 cells from tumors as described in **c**. Bar graphs depict mean percentage of PD-L1 positive Ag⁺ IGS cells plus SD, n=5 mice per group. Representative data of n=2 independent experiments are depicted. Two tailed Mann-Whitney U test was performed, with: p>0.9999 (ns); p=0.0317 (*); p=0.0079 (**).
Extended Data Fig. 3 | Distance from Ag⁻ IGS cells to the nearest Ag⁺ cell upon tumor cell co-injection. Analysis of the distance between Ag⁻ IGS tumor cells and the nearest Ag⁺ tumor cell for the imaging experiments depicted in Fig. 3. **a**, Representative image of a tumor with intermingled Ag⁻ and Ag⁺IGS cells. Scale bar is 100 μm. **b**, Plots show the min., max., and mean of 25th and 75th percentile plus the median for n=4 mice. **c**, Percentage of Ag⁺IGS reporter cells in the indicated distance bins to the nearest Ag⁺ cell, depicted mean plus SD for n=4 mice. Data obtained from three independent experiments.
Extended Data Fig. 4 | CD8+ T cell dependent Katushka signaling in IGS reporter cells in vivo. a, Flow cytometric analysis of Katushka expression in CFP+ IGS reporter (left panel) and GFP+ Ag+ (right panel) cells derived from mixed tumors described in Fig. 2b. Data from mice treated with HBSS are depicted in blue, data from mice treated with CDK4rL-specific CD8+ T cells are depicted in red, n=4 mice per condition, data obtained from one experiment. b, Representative images of tumors before and 120h after injection of CDK4rL-specific CD8+ T cells (left panel, three independent experiments with n=1 mouse each) or HBSS (right panel, two independent experiments with n=1 mouse each), for the imaging experiments described in Fig. 3. SHG: Second-harmonic generation. Scale bar is 200 μm.
Extended Data Fig. 5 | Analysis of T cell mediated loss of Ag-presenting tumor cells over time. a, Relative GFP+ volume in tumors from imaging experiments described in Fig. 2 quantified over time. Mean and SEM are depicted for n=5 mice (n=5 mice for time 0, 16, 24, and 32 h; n=4 for 40, 48, and 72 h; n=3 for 120 h) from data obtained in all independent experiments. b, The distance between CFP+ bystander tumor cells and the closest GFP+ Ag+ tumor cell was determined at indicated time points from tumors described in Fig. 2 for n=2 mice. Data are obtained from two independent experiments, boxplot presenting the minimum, 25th percentile, median, 75th percentile and the maximum For total sample size per timepoint see Source Data ED_Fig5_source table.
Extended Data Fig. 6 | CD8+ T cell quantification in Ag+ and Ag− IGS reporter tumor areas. a, Quantification of mOrange2+ CD8+ T cells in tumors with spatially separated GFP+ Ag+ (green) and CFP+ Ag− IGS reporter cell (cyan) islands obtained by sequential injection, as described in Fig. 4d, e. Number of mOrange2+ T cells was determined in multiple three-dimensional stacks of 2.5·107 μm3 in either Ag+ or Ag− areas. Symbols represent individual mice, and mean and SD for n=4 mice are depicted, obtained from two independent experiments. Normal distribution was confirmed by D’Agostino and Pearson omnibus normality test. Two tailed unpaired t-tests were performed, p=0.0003 (**). b, Estimate of the ratio of tumor cells to T cells in Ag+ and Ag− areas under the assumption that the diameter of an average tumor cell is 24 μm. c, Purified CD8+ T cells were activated with plate-bound anti-CD3/anti-CD28 antibodies for 2h. Subsequently, cells were either left untreated or were treated with 5nM LCKi inhibitor for the indicated times. Cells were washed to remove previously secreted IFNγ, and fresh control medium or medium containing 5nM LCK inhibitor was added to the cells. After 3h incubation, supernatants were collected and IFNγ concentrations were analysed. Bar graph shows mean IFNγ concentrations of n=3 technical replicates. Representative data of four independent experiments are depicted. d, As in c, depicting the IFNγ concentration in supernatants obtained from 2h LCK inhibitor treated cell cultures as a percentage of IFNγ concentration in control, non treated cell cultures. Dots represent four independent experiments, using different T cell donors in each experiment.
Extended Data Fig. 7 | Distinct β2m positive and negative areas in human cancers. a, Immunohistochemical staining of β2m and b, β2m and CD8 proteins on FFPE tissue of indicated human tumors. Heterogeneous β2m signal was observed in 16/51 tumors analyzed, one representative slide per tumor (obtained from resection material) was assessed and representative images are depicted in a. a. Scale bars are 100 μm. Note that CD8+ T cells in tumors predominantly localize to β2m high regions, representative images are depicted in b. Scale bars are 250 μm.
Extended Data Fig. 8 | CD8⁺ T cell mediated killing of bystander OVCAR5 tumor cells. A mixture of GFP⁺ Ag⁺, CFP⁺ Ag⁺ IFNγR proficient and CFP⁺ Ag⁺ IFNγR deficient OVCAR5 cells (2:1:1 ratio) was treated with CDK4r⁻/⁻ specific CD8⁺ T cells at a 2:1 T cell: tumor cell ratio, or left untreated, and cell survival was analyzed by staining with IR-Dye and subsequent flow cytometry. **a**, Representative plots depicting the percentage of IR-Dye⁺ cells for the indicated groups. **b**, Quantification of **a**, bar graph shows mean of n=3 technical replicates. Representative data of two independent experiments are depicted.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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Publicly available source data were not used in this study. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Sample size

No expected effect size was pre-specified. Generally accepted samples sizes were used, with a significant difference between conditions indicating that the sample size is sufficient.

Data exclusions

In the analysis of Figure 4f, areas smaller than 4×10^4 um^2 were excluded from analysis due to the very small numbers of cells present, and therefore high variability in the measurements. No further data were excluded from the analyses. Exclusion criteria were not established prior to the experiments.

Replication

Every figure states how many times each experiment had been repeated. To ensure experiments could be reliably reproduced, fully independent experiments were performed for most experiments, as defined by commonly accepted standards. All attempts at replication were successful.

Randomization

Treatment of tumor bearing mice [controls/ T cells] was randomized.

Blinding

Intratinal microscopy analysis was performed blinded, as such that the person performing the analysis was not aware of the time points corresponding to the data. Tumor analysis by flow cytometry were blinded with the person performing the analysis not being aware of the treatments given.

Reporting for specific materials, systems and methods

We require information from authors about some types of 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         |
|     | Animals and other organisms |
|     | Human research participants |
|     | Clinical data         |

### Methods

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

### Antibodies

- anti-human CD8 (APC; clone RPA-T8; BD biosciences; CAT#555369; Lot#8169667; dilution for Flow Cytometry 1:30)
- anti-human IFNγ/CD119 (PE; Clone 2B7-208; ebioscience; CAT#12-1199-41; Lot#42777721; dilution for flow cytometry 1:50)
- anti-human HLA-A*02 (APC; clone B37.2; BD Biosciences; CAT#51341; Lot#8208764; dilution for flow cytometry 1:50)
- anti-mouse TCRβ constant domain (PE; clone H57-597; BD Biosciences; CAT#553172; Lot#091623; dilution for Flow cytometry 1:150)
- anti-human-B2m (polyclonal, DAKO / Agilent; CAT# A0072; Lot#00086522; dilution for IHC 1:4500)
- anti-CD8 (clone CB/144B, DAKO; CAT# M7103; Lot# 20048132; dilution for IHC 1:200)
- anti-PD-L1/CD274 (APC; clone MIH1; ebioscience; CAT#17-5983-42; Lot#12159-1634; dilution for Flow cytometry 1:150)

### Validation

All antibodies were validated for their application by the manufacturer (see above in ‘Antibodies used’ for details). All anti-human antibodies are validated on human material, the anti-mouse antibody is validated on mouse material.

### Eukaryotic cell lines

Sources of all the cell lines is indicated in the methods section. OVCARS cells were kindly provided by Dr. F. Scheeren. MDA-MB-231 cells and HEK293T cells were purchased from American Type Culture Collection (ATCC no. CRL-3216 and HTB-26). FLYD18 packaging cells were obtained from European Collection of Authenticated Cell Cultures (ECACC no. 95091902)

OVCARS cells have been validated by STR analysis. MDA-MB-231, HEK293T and FLYD18 cells were directly obtained from ATCC and ECACC and have not been further validated.

Cell lines have been tested negative for Mycoplasma contamination.
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Species: Mice  
|                    | Strains: NOD-scid IL2rnullIL2rnull  
|                    | Sex: male and female (for mammary fatpad tumor injections only female mice were used)  
|                    | Age: 8-26 weeks old  

| Wild animals       | This study did not involve wild animals  
| Field-collected samples | This study did not involve samples collected from the field  
| Ethics oversight   | All animal experiments were approved by the Animal Welfare Committee of the Netherlands Cancer Institute, in accordance with national guidelines  

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

Human research participants

Policy information about studies involving human research participants

| Population characteristics | No data on specific patient characteristics. 51 samples collected from patients undergoing tumor resection surgery were assessed for 82m and CD8 expression.  
| Recruitment                | Patients were not recruited, material was obtained following an opt-out procedure.  
| Ethics oversight           | Study was approved by the local medical ethical committee [institutional review board (IRB) of The Netherlands Cancer Institute, Antoni van Leeuwenhoek hospital (NHL-AVL)].  

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

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 lines were cultured, and when attaching cells, harvested by trypsin EDTA treatment. All cells were stained in PBS containing 0.5% w/v bovine serum albumin and 0.2% w/v sodium azide.  
| Instrument         | BD LSR II or BD Fortessa  
| Software           | Data were collected using BD FacsDiva and analyzed using FlowJo  
| Cell population abundance | All sorted populations described in the text were sorted with >90% purity, as confirmed by flow cytometry after cell outgrowth to high numbers.  
| Gating strategy    | Cells were gated first by morphology to exclude cell debris, doublets were then gated out by FSC-A/FSC- followed by exclusion of dead cells by gating on IR-Dye negative cells.  

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