A fluorogenic probe for granzyme B enables in-biopsy evaluation and screening of response to anticancer immunotherapies

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Immunotherapy promotes the attack of cancer cells by the immune system; however, it is difficult to detect early responses before changes in tumor size occur. Here, we report the rational design of a fluorogenic peptide able to detect picomolar concentrations of active granzyme B as a biomarker of immune-mediated anticancer action. Through a series of chemical iterations and molecular dynamics simulations, we synthesize a library of FRET peptides and identify probe H5 with an optimal fit into granzyme B. We demonstrate that probe H5 enables the real-time detection of T cell-mediated anticancer activity in mouse tumors and in tumors from lung cancer patients. Furthermore, we show image-based phenotypic screens, which reveal that the AKT kinase inhibitor AZD5363 shows immune-mediated anticancer activity. The reactivity of probe H5 may enable the monitoring of early responses to anticancer treatments using tissue biopsies.
The response of CD8+ T cells is one of the main immune mechanisms to protect the human body against cancer. The presence of CD8+ T cells in tumors is indicative of a favorable prognosis in cancer patients1–6; however, there is high variability in how patients respond to immunotherapies7–9. Clinical imaging can measure the size of tumors and, to some extent, the infiltration of CD8+ T cells but they cannot provide readouts on how efficiently the immune system is responding against cancer cells. This limitation hinders the evaluation of drugs and the personalized optimization of immunotherapies to minimize off-target toxicity10–13. Chemical approaches that directly measure the activity of tumor-infiltrating T cells will accelerate the screening and optimization of anticancer drugs as well as improving the monitoring of early responses to treatment in a personalized manner.

Standard methods for in vitro monitoring of CD8+ T cell cytotoxicity employ lactate dehydrogenase (LDH), tetrazolium dye-based metabolic assays (MTS, MITT) or 31Cr release assays14–16. These assays report bulk cytotoxicity rather than T-cell-specific immune responses. The activity of CD8+ T cells can be indirectly monitored by measuring the concentration of extracellular cytokines17 and membrane proteins (e.g., CD107a) using antibodies18. These methods assess T cell function but do not directly report on cancer cell death, thus are not useful biomarkers of the immune killing capacity in tumors. The chemical design of activity-sensing reporters of granzyme B (GzmB) represents a potentially effective strategy for monitoring the cytotoxic activity of CD8+ T cells in cancer. GzmB is a serine protease that is stored inactive in T cells until antigen-driven recognition prompts its release and activation inside cancer cells. Probes for detecting GzmB include antibodies and fusion proteins, but these do not distinguish between the active and inactive forms of the enzyme19, and activatable constructs based on the Ile-Glu-Pro-Asp (IEPD) sequence first described by Thornberry et al.20. Some examples of the latter include poly-lysine graft copolymers for myocarditis21, contrast agents for preclinical imaging22–24, and nanoprobe for urinalysis25,26. These probes rely on a chemical scaffold with limited reactivity (e.g., Vmax in the range of pmol min⁻¹ and kcat/KM ratios around mM⁻¹ s⁻¹, Table 1).

In this work, we rationally design GzmB substrates to identify peptide sequences with an optimized fit into the active site of the enzyme, suggesting that an alternative mode of binding can be exploited for the design of high-affinity probes for GzmB. Following development into FRET constructs we furnish probe H5, which exhibits kcat/KM ratios several orders of magnitude greater than those observed for IEPD tetrapeptides. We then use probe H5 to enable real-time measurements of the anticancer activity of T cells in a mouse model of immune-mediated tumor regression. We also deploy H5 in image-based screens to identify drugs able to reinvigorate CD8+ T cell activity against cancer cells. Finally, we optimize the application of probe H5 in tissues from lung cancer patients to detect in situ T cell cytotoxic activity in human tumors.

| Compound | Amplification mechanism | Kcat (s⁻¹) | KM (µM) | Kcat/KM (M⁻¹ s⁻¹) | LoD | Ref. |
|----------|------------------------|------------|---------|-------------------|-----|------|
| 4-mer    |                        |            |         |                   |     |      |
| Ac-IEPD-AMC | Fluorogenic dye | 0.5        | 160     | 3.3 × 10³         | 25 nM | Ref. 39 |
| PEGylated IONPs | Nanoconstruct | -0.002    | -0.2    | 1.1 × 10⁴         | n.d. | Ref. 25 |
| CyGbp | Fluorogenic dye | 0.07       | 22.7    | 3.1 × 10³         | n.d. | Ref. 26 |
| T1 | FRET | 2.3        | 30.8    | 5.9 × 10⁴         | 17 nM | This work |
| 6-mer    |                        |            |         |                   |     |      |
| H5 | FRET | 1/7        | 9.6     | 1.2 × 10⁷         | 6 pM | This work |
| 9-mer    |                        |            |         |                   |     |      |
| qTFJ | FRET | 0.65       | 9.9     | 6.6 × 10⁵         | n.d. | Ref. 27 |

Table 1 Summary of fluorescent peptide-based probes for GzmB.
**Fig. 1** The hexapeptide H5 achieves high reactivity and selectivity for GzmB by accessing a unique binding pocket. 

(a) Fluorogenic hexapeptide H5 and fluorescence quantum yields of intact and cleaved probe. 
(b) Tetra- and hexapeptide sequences, fluorescence increases and t\textsubscript{50} values upon incubation (25 µM) with hGzmB (20 nM). Data as means ± SEM (n = 3). 
(c) Time-course fluorescence of H5 (25 µM, green, 510 nm) and Ac-IEPD-AMC (25 µM, blue, 450 nm) after incubation with hGzmB (20 nM) at 37 °C. Probe H5 alone (25 µM, black, 510 nm). Data as means ± SEM (n = 5). 
(d) Cleavage rate of probe H5 by hGzmB (20 nM) as a function of substrate concentration. Data as individual replicates and kinetic values determined using the Michaelis-Menten equation (n = 3). 
(e) Fluorescence changes of probe H5 (25 µM) after incubation with proteases (20 nM) at 37 °C for 60 min. Data as means ± SEM (n = 3). For active GzmB vs pro-GzmB, p < 0.0001. For active GzmB vs GzmB+ inhibitor, p = 0.0003. For active GzmB vs GzmA, p < 0.0001. 
(f) Representative binding mode of IEPD-Dabcyl in T1 (left) and IEPDAL-Dabcyl in H5 (right) from the MD simulations. 
(g) Detailed interactions at P1’ and P2’ sites for the probe H5 with overlaid structures from 3 independent runs. P\textsubscript{-}values from two-tailed t-tests. Source data (b–e) provided as a Source Data file.
conversion whereas the peptide H5 showed the highest reactivity, with fast and complete cleavage in less than 30 min (Table S2 and Fig. S2). Interestingly, this result suggested that a smaller amino acid (i.e., alanine instead of tryptophan) was better tolerated by the enzyme. To confirm this hypothesis, we synthesized negative control peptides of the H4 and H5 sequences where we included an arginine either in P1 or P2, given that the hGzmB crystal structure features one arginine (Arg226) in the S1 subsite and electrostatic repulsion between arginine residues should disfavor binding.33 The resulting hexapeptides H6 (IEPDWR) and H7 (IEPDLR) showed markedly reduced reactivity (i.e., 40 and 60% conversion, respectively, Table S2), yet still much higher reactivity than the tetrapeptide T1. These results suggest that FRET hexapeptide constructs react with hGzmB much faster than shorter tetrapeptides. We prepared all peptides using solution and solid-phase synthesis (Supplementary Note 1), isolated them by preparative HPLC in purities over 95%, and confirmed their identity by high-resolution mass spectrometry (Table S3).

Probe H5 is a highly specific substrate of human GzmB by accessing a unique binding pocket. We analyzed the fluorogenic response of all hexapeptides H1-H7 by measuring their fluorescence emission in the presence of recombinant hGzmB (Fig. 1a). As expected, all hexapeptides outperformed the tetrapeptide T1, with signal-to-background ratios ranging from 12-fold (for peptide H6) to 153-fold (for peptide H5) (Fig. 1b and S3). We also measured the time taken for the most reactive compounds to reach 50-fold fluorescence increase (termed t50). Probe H5 showed the fastest response among all peptides with a t50 of 7 min (Fig. 1b, c). We also compared the kinetic properties of probe H5 to previously reported tetrapeptide constructs and the best three shorter tetrapeptides. We prepared all peptides using solution and solid-phase synthesis (Supplementary Note 1), isolated them by preparative HPLC in purities over 95%, and confirmed their identity by high-resolution mass spectrometry (Table S3).

Probe H5 detects real-time reinvigoration of T cells in cocultures with cancer cells. Given the reactivity and selectivity of probe H5 for GzmB, we studied its utility to measure the cytotoxic activity of T cells during the attack on cancer cells. To investigate this, we co-cultured mouse CD8+ T cells and E0771 mammary tumor cells (Fig. 2a). Substrates for mouse GzmB feature phenylalanine in P2—instead of proline in human substrates46—and, yet we observed a good response of the probe H5 to mouse GzmB, with slightly better reactivity than the hexapeptide analogue containing phenylalanine (probe H5α-IEPD-CHO, Fig. S5). Therefore, we decided to use probe H5 for both mouse and human assays. First, we optimized the co-cultures to effectively increase the levels of GzmB in CD8+ T cells. Incubation with interleukin IL-2, an essential cytokine for the survival, proliferation, and activation of CD8+ T cells, produced the largest reinvigoration, with over 80% CD8+ T cells expressing GzmB after IL-2 treatment (Fig. S6) and confirmation by fluorescence microscopy using anti-GzmB (Fig. 2b). Next, we applied these conditions to co-cultures of CD8+ T cells and genetically modified E0771-La2-NLR cells, which express the red fluorescent protein mKate47 to facilitate their detection and incubated them with probe H5 and Annexin V-AF647, a marker of apoptosis. Flow cytometric analysis revealed that >80% of cancer cells that had been cultured with activated T cells were double-stained with probe H5 and Annexin V-AF647, confirming that the emission of probe H5 reports immune-mediated cancer cell death (Fig. 2c and Fig. S7). Co-cultures of cancer cells with non-reinvigorated T cells or cancer cells treated with staurosporine—a relatively nonspecific kinase inhibitor that induces apoptosis—showed weak fluorescence, confirming that probe H5 only detects dead cancer cells that have been killed by CD8+ T cells and not all dead cancer cells (Fig. 2c and Fig. S7). Furthermore, we observed that the fluorescence signals of probe H5 were significantly reduced upon blockade with the GzmB inhibitor Ac-IEPD-CHO (Fig. S7). Microscopy experiments also corroborated that probe H5 only stained the cytoplasm of mKate+ cancer cells, but not T cells
Fig. 2 Probe H5 detects GzmB-mediated anticancer activity of CD8+ T cells. a Schematic procedure for co-culture assays. b Representative microscopy images of CD8+ T cells before/after reinvigoration and staining by anti-GzmB (10 nM, red) and Hoechst 33342 (1 µM, blue) (n = 3). Scale bar: 10 µm. c Flow cytometry (gating: Fig. S7) of E0771 cells in co-culture with active (IL-2, 24 h) or inactive (IL-2, 2 h) CD8+ T cells, or alone with staurosporine (1 µM, 1 h). Legends: viable (gray), H5-stained (green), apoptotic (red). H5 (5 µM), Annexin V-AF647 (10 nM). Data as means ± SEM (n = 3). d Confocal microscopy of mKate-expressing E0771 cancer cells (red) stained with H5 (25 µM, green) in co-culture with active T cells (left), non-active T cells (centre), or active T cells plus Ac-IEPD-CHO (right). Black arrows highlight T cells, yellow arrows highlight H5-stained intracellular GzmB puncta. Quantification of fluorescence intensity by image analysis shown in Fig. S8. Scale bar: 10 µm. e Time-course fluorescence microscopy of OT-I CD8+ T cells stained with Cell Tracker Orange (red) killing OVA-EL4 cancer cells in the presence of H5 (10 µM, green) and Sytox Blue (1 µM, blue) (n = 3). Scale bar: 10 µm. f Flow cytometric analysis of OVA-EL4 cancer cells from experiments in e. Data as means ± SEM (n = 3). P-values from two-tailed t-tests. Source data (c, f) provided as a Source Data file.
(Fig. 2d and Fig. S8). As additional controls, we observed that probe H5 was not cytotoxic (Fig. S9) and did not stain cancer cells in co-cultures where T cells were inactive or in co-cultures that had been pretreated with the reversible GzmB inhibitor Ac-IIEPD-CHO (Fig. 2d). Furthermore, we synthesized an ‘always-on’ derivative of probe H5 (H5-unquenched) to confirm its permeability in different cancer cell lines (Fig. S10). Altogether, these results confirm that probe H5 can detect cancer cell death resulting from the attack of invigorated CD8+ T cells and suggest that the fluorescence emission of probe H5 can be used as a biomarker of immunomodulatory efficacy in live cultures.

Next, we examined whether H5 could be used to image how CD8+ T cells attack cancer cells in real-time with an antigen-specific method of T cell activation. We utilized CD8+ T cells expressing the OT-I transgenic receptor, which specifically targets cells presenting the OVA-derived SIINFEKL antigen in the context of H-2k and co-cultured them with SIINFEKL-pulsed EL4 cancer cells (Fig. S11). T cells were counterstained with Cell Tracker Orange for ease of identification and incubated with probe H5 and the cell death marker Sytox Blue before time-lapse imaging. Initially, the probe H5 was silent but its emission inside target cells gradually increased as CD8+ T cells started to form immune synapses with cancer cells (Fig. 2e and Movie S1 and S2). After several contacts between CD8+ T cells and cancer cells, the green fluorescence signal of H5 colocalized with the blue signal of Sytox Blue, indicating that targeted cancer cells had undergone GzmB-mediated apoptosis. Quantitative flow cytometric analysis also confirmed that the fluorescence signal of the probe H5 in cancer cells preceded that of Sytox Blue, which corroborates active GzmB as an early biomarker of immunemediated cancer cell death (Fig. 2f). In parallel, we performed in vitro experiments mimicking aspects of the intracellular environments found in cancer cells that receive multiple contacts—and thus multiple deliveries of GzmB—from CD8+ T cells and observed an accumulative fluorogenic signal for probe H5 to spiked 1 nM increments of GzmB (Fig. S12).

**Probe H5 detects T-cell-mediated tumor regression in a mouse model of cancer.** We further investigated the capability of the probe H5 to detect GzmB activity in a mouse model of tumor regression. Serrels et al reported that inhibition of Focal Adhesion Kinase (FAK) can drive T-cell-mediated regression of squamous cell carcinoma (SCC) tumors via modulation of the immunosuppressive microenvironment (Fig. 3a, b), with FAK inhibitors currently tested in clinical trials in combination with immune checkpoint inhibitors19. Because the tumor regression in SCC FAK (−/−) mice is dependent on CD8 T+ cells, this preclinical model represented an excellent platform to examine whether the probe H5 could detect T-cell-mediated cancer cell death in tumors. First, FVB immune-competent mice were challenged with SCC FAK (−/−) cancer cells or wild-type SCC cancer cells (as a negative control), and tumors were grown for 2 weeks. As expected, SCC FAK (−/−) tumors were significantly smaller and contained fewer live cancer cells (Fig. 3c) than wild-type SCC tumors. The number of CD8+ T cells was around 10-fold higher in SCC FAK (−/−) tumors, implying an invigorated T cell response (Fig. 3d), which also showed higher expression levels of GzmB as determined by ELISA (Fig. S13). In order to evaluate whether the cancer cells in SCC FAK (−/−) tumors contained intracellular active GzmB, tumors were harvested and treated with probe H5 for 30 min before being analyzed by flow cytometry. Notably, over 40% of cancer cells in the SCC FAK (−/−) tumors were labeled with probe H5, whereas wild-type SCC tumors were almost not stained (Fig. 3e, f). Furthermore, we observed that the fluorescence signal of the probe H5 was exclusively found in a subset of cancer cells but not in other cells found in tumors (e.g., monocytes, fibroblasts, CD4+ T cells) (Fig. 3g, h and Fig. S14). Altogether, these results corroborate the utility of probe H5 to rapidly detect T-cell-mediated cell death in mouse tumors.

**Probe H5 identifies immunomodulatory activity in drug screens and human tumors from lung cancer patients.** We next assessed the utility of the probe H5 in screens of immunomodulatory drugs and clinical assays in human tumors. First, we adapted our co-cultures of mouse CD8+ T cells and E0771 cancer cells to a 384-well plate format for image-based phenotypic assays that could screen small molecules for invigorating the killing capacity of CD8+ T cells (Fig. 4a). We used the ImageXpress high-content analysis system to test a collection of anticancer drugs with varied pharmacological targets (Table S5). E0771 and CD8+ T cells were co-cultured for 2 days and then incubated with IL-2 (100 U mL−1) and each individual drug at their respective working concentrations for 3 h (Table S5). One hour before imaging, the probe H5 was added to the wells and we acquired fluorescence microscopy images, including Hoechst 33342 as a nuclear counterstain. The fluorescence intensity of probe H5 inside cancer cells was used to compare the immunomodulatory capacity of all 44 drugs. We included wells with a high concentration of IL-2 (250 U mL−1) as a positive control for high GzmB and wells with 100 U mL−1 IL-2 plus rapamycin, a known mTOR inhibitor that blocks IL-2-induced activation of T cells, as a negative control for low GzmB activity. Of note, a small set of compounds with different pharmacological functions (e.g., protein kinase inhibitors, inhibitors of microtubule function, DNA alkylating agents, proteasome inhibitors) exhibited superior staining than the single treatment with IL-2, indicating the ability to invigorate anticancer T cell activity (Fig. 4b; for an extended description of the screening results, see Supplementary Note 2). Among these, the AKT kinase inhibitor AZD5363 (1 μM) exhibited the brightest H5 fluorescence staining and was selected for further studies. We analyzed the cell viability and extent of H5 fluorescence staining in E0771 cancer cells that had been incubated with AZD5363 as well as in CD8+ T cell plus E0771 cell co-cultures that were incubated with IL-2 only or IL-2 plus AZD5363. The treatment of AZD5363 on its own did not induce significant cancer cell death or led to brighter fluorescence labeling by the compound H5, whereas the same concentration of AZD5363 in combination with 100 U mL−1 IL-2 caused significant cancer cell death and H5 fluorescence emission in CD8+ T cell-E0771 cell co-cultures (Fig. S16). These observations are in agreement with recent reports that suggest AKT inhibition may prevent CD8+ T cell exhaustion, resulting in enhanced cytolytic activity against target cells50. These results highlighted the potential of AZD5363 to invigorate the anticancer activity of CD8+ T cells when used in combination with IL-2 and demonstrate the utility of our H5-based imaging screen platform for the identification of immunomodulatory drugs.

Finally, we examined whether probe H5 could also monitor cytotoxic T cell function in biopsies from lung cancer patients as a potential method to screen their predisposition to anticancer treatments. For these experiments, we obtained paired (i.e., tumor and non-tumor, representative images displayed in Fig. 4c) tissue resections from five treatment-naïve lung cancer patients undergoing surgical resection. To analyze whether probe H5 could detect ongoing GzmB-mediated killing of tumor cells, we treated all samples with probe H5 (30 min, r.t.) and surface markers for epithelial cells (EpCAM), leukocytes (CD45), and cytotoxic T lymphocytes (CD8) before flow cytometry analysis. As shown in
Fig. 3 Probe H5 detects T-cell-mediated tumor regression in a mouse model of squamous cell carcinoma. a Experimental timeline of the CD8+ T-cell-mediated tumor regression model. b Cell populations found in wild-type SCC and SCC FAK (−/−) tumors. c–f SCC and SCC FAK (−/−) cells were injected into FVB immunocompetent mice (1 × 10⁶ cells/mouse) and tumors were harvested on day 14. Flow cytometry (gating: Fig. S15) of wild-type SCC and SCC FAK (−/−) tumors for cell viability with live/dead stain (c); CD8+ T cell infiltration with anti-CD8-PE (p < 0.0001) (d); percentage of GzmB-positive SCC cancer cells by staining with H5 (5 μM, 30 min) (p = 0.0042) (e); fluorescence intensity of H5 staining inside SCC cancer cells (525 nm) (p = 0.0108) (f). Data in c–f as means ± SEM (n = 4) (p = 0.0005). g, h SCC FAK (−/−) tumors (g) and wild-type SCC tumors (h) (ex vivo stained with 5 μM compound H5) were analyzed by flow cytometry and presented as pseudo-colored two-dimensional tSNE (t-distributed stochastic neighbor embedding) plots to determine the distribution of the probe in different cell populations (left) and the fluorescence intensity of H5 staining (right) (n = 4). P-values from two-tailed t-tests using Welch correction. Source data (c–f) provided as a Source Data file.
**Fig. 4** Probe H5 detects immunomodulatory action in phenotypic screens and T cell cytotoxic activity in human tissue from lung cancer patients.

a) Experimental protocol of the phenotypic screen. b) Fluorescence intensity of probe H5 in co-cultures of E0771 cells and IL-2-activated CD8+ T cells after incubation with small molecules (C1-C44). Wells received 100 U mL⁻¹ IL-2 (low IL-2) for T cell viability. High IL-2 (250 U mL⁻¹) used as a positive control for invigorated CD8+ T cells and rapamycin (0.3 μM) used as a negative control. Probe H5 (20 μM) and Hoechst 33342 (1 μM) were incubated for 1 h and fluorescence images were acquired with an ImageXpress™ XLS. Data as means ± SEM (n = 8). Chemical structures of drugs showing H5 fluorescence signals above those with 250 U mL⁻¹ IL-2. c) Representative H&E microscope images of non-tumor (left) and lung adenocarcinoma (right) paired samples. Scale bar: 50 μm. d) Cytometry analysis (gating: Fig. S17) showing the percentage of EpCAM+ H5+ epithelial cells found in paired tissues (non-tumor vs tumor) from lung cancer patients after incubation with probe H5 (5 μM). Data as means ± SD (n = 5). P-value from two-tailed t-tests. Source data (b, d) provided as a Source Data file.
Fig. 4d, we observed a significant increase in the percentages of probe H5+ cells within the EpCAM + population in cancer tissue across all patients, indicative of the cytotoxic activity of CD8+ T cells against lung epithelial cells in tumors. The short excitation and emission wavelengths of BODIPY-FL might be limiting for some in vivo imaging studies, therefore further optimization studies will be needed to adapt probe H5 for the evaluation of immunotherapy regimes in cancer patients. Altogether, our results indicate that probe H5 can be used in vitro in high-throughput screening assays to facilitate the discovery of immunotherapy combinations as well as in the clinical characterization of human tumor biopsies, opening new avenues to accelerate the development of personalized anticancer immunotherapies.

**Discussion**

Fluorogenic peptides are excellent scaffolds to develop agents monitoring protease activity. We here describe the rational design of a fluorogenic probe for rapid detection and imaging of active mouse and human GzmB, a key protease involved in initiating apoptosis of infected or malignant cells. Starting from the generic IEPD sequence, we have built a collection of FRET probes to optimize the hexapeptide H5 with an unprecedented K_{cat}/K_{M} ratio of 1.2 × 10^{7} M^{-1} s^{-1} and a limit of detection of 6 pM. Probe H5 displays a limit of detection within the physiological levels of GzmB found in human tissues and fluids (e.g., serum, synovial fluid), therefore it holds potential for the diagnosis and monitoring of inflammatory diseases (e.g., arthritis or inflammatory bowel diseases, among others)\(^{31−33}\). Furthermore, we have used molecular dynamic simulations to investigate the binding modes of the peptides and observed that the probe H5 binds to an extension of the catalytic cleft delineated by the loop 74–84 and the β-barrel 35–114 of hGzmB, which is not accessible by tetrapeptide sequences. The discovery of this alternative binding mode to active hGzmB may facilitate the design and optimization of future imaging probes and enzyme inhibitors, which could be key in combating some of the damaging autoimmune functions of GzmB\(^{81}\). We have demonstrated that the fluorescence emission of H5 can be used as a direct reporter of immune-mediated tumor-killing ability in live cultures of CD8+ T cells and cancer cells, both qualitatively by fluorescence microscopy and quantitatively by flow cytometry. Importantly, the probe H5 does not fluoresce inside T cells, where GzmB is inactive, or in cancer cells when killed by other agents (i.e., staurosporine) that are not related to immune-mediated cancer cell death. This selectivity profile of probe H5 may find application for monitoring GzmB activity in other diseases where the activation state of immune cells is currently under investigation, such as Epstein-Barr virus-mediated multiple sclerosis and chronic obstructive pulmonary disease\(^{4,5}\). We have also shown that the probe H5 can identify mouse tumors undergoing immune-mediated regression in a model of squamous cell carcinoma. This feature may enable future rapid analyses of immunity in solid tumors, as tumor growth and immune infiltration might be difficult to distinguish by some clinical imaging modalities. Probe H5 will also create avenues to design discovery platforms for identifying small molecule drugs invigorating immunomodulatory responses in image-based phenotypic screens. Finally, we have demonstrated the use of probe H5 for the clinical analysis of human tumor biopsies, highlighting a potential application for the personalized detection of early responses to anticancer immunotherapies.

**Methods**

**Chemical synthesis.** Full details of synthetic procedures and chemical characterization are included in the Supplementary Information.

**Fluorescence assays with recombinant enzymes.** Fluorescence-based assays with different enzymes were performed in the appropriate buffers. Probes (25 μM) were added to enzymes (20 nM or at the indicated concentration) in 384-well plates and their fluorescence emission was recorded at 450 nm (for AMC) or 510 nm (for BODIPY) at 37 °C using a Synergy H1 Hybrid spectrophotometer. In experiments with Ac-IEPD-CHO (50 μM), the inhibitor was preincubated for 1 h with GzmB before the addition of any probe.

**Primary cell isolation and cell culture.** Highly metastatic derivative of E0771 mouse mammary tumor cells expressing the nuclear-localized red fluorescent protein mKate (obtained from Dr Takanori Kitamura and Professor Jeffrey Pollard at The University of Edinburgh) were grown using Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U mL\(^{-1}\) penicillin and 100 mg mL\(^{-1}\) streptomycin), and 2 mM L-glutamine in a humidified atmosphere at 37 °C with 5% CO\(_2\). Cells were regularly passaged in T-25 cell culture flasks upon reaching 90% confluency. Primary CD8+ T cells were isolated from mouse spleens (Strain: C57BL/6, Sex: female, Age: 10–14 weeks, obtained from Charles Rivers) by tissue homogenization, erythrocyte lysis, and purification with magnetic beads (CD8 Microbeads Kit).

**Flow cytometry.** E0771 cells (5 × 10^4 cells/well) were plated on Geltrex-coated six-well plates with IL-2 (1000 U mL\(^{-1}\)) and co-cultured with either murine CD8+ T cells (2.5 × 10^5 cells/well) which had been previously cultured for 2 days in E-DMEM with anti-mouse CD3e (2 μg mL\(^{-1}\), ThermoFisher, Catalog: 16-0031-86), anti-mouse CD28 (5 μL mL\(^{-1}\), Thermofisher, Catalog: 16-0281-86), and IL-2 (80 U mL\(^{-1}\)). Treatments with staurosporine (1 μM) were for 1 h. Flow cytometry preparation included treatment with trypsin-EDTA (0.05%), wash, resuspension in PBS and incubation with probe H5 (5 μM) for 1 h at 37 °C. Cells were then washed twice and incubated with Annexin V-AF647 (10 nM, 1:100 dilution) prior to flow cytometry analysis in a 5 L LSII automated flow cytometer. For the study with the GzmB reporter (protein mKate) obtained from Dr Takanori Kitamura and Professor Jeffrey Pollard, the purified mKate (obtained from Dr Takanori Kitamura and Professor Jeffrey Pollard) was labeled with antibodies (1:100 dilution) and the probe H5 for 2 h. Flow cytometry was performed with FlowJo V10. Excitation/emission wavelengths: H5 (488 nm, 525 ± 50 nm), mKate (561 nm, 635 ± 15 nm), Annexin V-AF647 (640 nm, 670 ± 14 nm).

**Live-cell fluorescence confocal microscopy.** E0771 (1.5 × 10^5 cells/well) were plated on Geltrex-coated 8-chamber glass slides with freshly isolated murine CD8+ T cells (6 × 10^4 cells/well) in enriched E-DMEM phenol red-free media with IL-2 (80 U mL\(^{-1}\)), anti-mouse CD3e (5 μg mL\(^{-1}\), Thermofisher, Catalog: 16-0031-86) and anti-mouse CD28 (2 μg mL\(^{-1}\), Thermofisher, Catalog: 16-0281-86). After 48 h, cell reinvigoration was performed by the addition of IL-2 (1000 U mL\(^{-1}\)) and incubation for 3 h. Probe H5 was used at 25 μM and Ac-IEPD-CHO at 50 μM with 1 h preincubation. After 1 h treatment at 37 °C with H5, cells were washed with media and imaged under a Leica TCS SP8 fluorescent confocal microscope (software: Leica Application Suite X) under a x40 oil objective. Excitation wavelengths: 488 nm (H5), 561 nm (mKate). Images were analyzed with ImageJ.

**Preclinical cancer model.** All animal work was carried out in compliance with UK Home Office guidelines. SCC tumors were established and labeled for flow cytometry as previously described\(^7\). Briefly, 1 × 10^6 SCC cancer cells obtained from Dr Alan Serrels at The University of Edinburgh were injected subcutaneously into FVB/N mice (n = 4 for both WT and FAK (−/−) groups, Sex: female, Age: 10–14 weeks, obtained from Harlan UK), which were sacrificed 14 days post-implantation and tumors disaggregated to generate a single-cell suspension. Cells were labeled with antibodies (1:100 dilution) and the probe H5 for 30 min at r.t. and analyzed by flow cytometry. Data analysis was performed using FlowJo V10 software.

**Drug screening.** E0771 (750 cells/well) and freshly isolated murine CD8+ T cells (6000 cells/well) were plated in 384-well plates as described above. After 2 days of culture at 37 °C, cells were further incubated with IL-2 alone (100 or 250 U mL\(^{-1}\)) or IL-2 (100 U mL\(^{-1}\)) in combination with drugs (Table S5) for 3 h. Compound H5 (20 μM) were added for 1 h at 37 °C, cells were washed with PBS and imaged under ImageXpress\(^\text{TM}\) XLS using KRB (10% FBS) buffer as media. Fluorescence images (4 sites/well) were acquired under a x40 objective using 405 nm (Hoechst) and 488 nm (H5) excitation wavelengths and data were analyzed with MetaXpress 3 software using the Custom Module Editor. Briefly, nuclei were used to create a pseudo-cell area and GzmB-positive puncta were detected using a top-hat filter (pixel size: 10, circle shape) followed by an average filter (3 × 3 pixels).
Human tumors. Tissue samples of tumor and paired non-tumor (n = 5 for both conditions) lungs were taken from treatment-naive patients undergoing surgical resection. Participants provided written informed consent and the study was approved by NHS Lothian REC and facilitated by NHS Lothian SAHSC Biorepository (REC No: 15/ES/0094). Fresh samples were minced, incubated with 1 mg mL⁻¹ collagenase IV, 1 mg mL⁻¹ DNase I, 50 μU mL⁻¹ hyaluronidase in DMEM for 1 h at 37 °C with agitation, and passed through 100 and 70 μm filters followed by red cell lysis. Single-cell suspensions were washed in PBS and stained with Zombie UV prior to surface staining for EpCAM-BV650 (1:100, BioLegend, Catalog: 344748) for 30 min at r.t. in the presence or absence of probe B. Cells were permeabilized twice in PBS 2% fetal calf serum (FCS) prior to collection. For microscopy, formalin-fixed paraffin-embedded slides of non-small cell lung cancer and paired non-cancerous lung were deparaffinized, rehydrated, stained with hematoxylin and eosin, followed by dehydration and imaging on a Zeiss Axiolab microscope.

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

Data availability Models were built with Maestro and AMBER16 using PDB id. 1IAU (https://www.ebi.ac.uk/pdbe/entry/pdb/iaua). The data from Figs. 1–4 and Supporting Info Fig. 1–19 of this study are available from the corresponding authors upon request. Source data are provided with this paper.

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Author contributions
J.I.S. synthesized chemical compounds; J.I.S., L.M.T., D.G., E.J.T., and Z.C. performed experiments in vitro and ex vivo; A.B.-B. and E.W.R. performed time-lapse microscopy experiments; J.J.J. and J.M. performed computational studies; I.J.V. and M.V. obtained samples, designed, and performed experiments with human biopsies; J.I.S. and M.V. conceived the project, analyzed and discussed the results, and wrote the paper with contributions from all authors; M.V. supervised the overall project.

Competing interests
The University of Edinburgh has filed a patent covering some of the technology described in this manuscript. J.M. is a current member of the Scientific Advisory Board of Cresset. The remaining authors declare no competing interests.

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