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A cell-engineered system to assess tumor cell sensitivity to CD8^{+} T cell-mediated cytotoxicity

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ABSTRACT

In vitro assays that evaluate CD8^{+} T cell-mediated cytotoxicity are important to aid in the development of novel therapeutic approaches to enhance anti-tumor immune responses. Here, we describe a novel cytotoxicity co-culture assay that circumvents the problem of highly variable allogeneic responses and obviates the constraints of HLA-restriction between effector and target cells. We show that this assay can be easily applied to a panel of tumor cell lines to provide additional insights into intrinsic drivers of sensitivity/resistance to T cell-mediated killing, and to evaluate the impact of targeted therapies on both tumor and T cell compartments.

Introduction

Immunotherapy is now established as a standard of care for many cancer types.[1,2] Despite this rapid progress, a greater understanding of intrinsic or adaptive mechanisms of resistance to immunotherapy is required to increase the proportion of responding patients, and durability of responses. Furthermore, there is now a more urgent need to better characterize how targeted therapies, designed to specifically affect tumor cells, may impact the anti-cancer immune response to identify productive combinations and avoid potentially antagonistic drug combinations.

CD8^{+} cytotoxic T cells are considered as the principal cellular mediators of anti-tumor immunity due to their capacity for specific recognition of tumor-associated antigens (TAA) and intrinsic cytotoxic potential.[3] Therefore, *in vitro* assays that permit evaluation of T cell cytotoxicity against tumor cells can be used to determine mechanisms of sensitivity/resistance and to screen novel agents that may modulate the outcome of the T cell/tumor cell interaction, providing important translational insights. T cells recognize peptide antigens in the context of the major histocompatibility complex (MHC) through their T cell receptors (TCR) providing 'signal 1'. Costimulatory proteins provide 'signal 2', via the CD28 co-receptor protein expressed on the surface of T cells. Activation of CD8^{+} cytotoxic T lymphocytes (CTLs) leads to target cell apoptosis through the release of lytic granules or via alternative mechanisms such as the Fas pathway, or cytokine release.[4] As such, most established *in vitro* T cell cytotoxicity assays depend on T cell antigen-specific recognition via tumor MHC-TCR engagement, and thus are either human leukocyte antigen (HLA) restricted or rely on alloreactivity.[5,6] Autologous assays are challenging given the

limitations on the numbers of HLA-matched donor T cells that can be sourced.[7] Moreover, allogeneic assay systems are intrinsically variable from donor to donor.[8] Therefore, it is difficult to assess a T cell response across a range of tumor cells with distinct genotypes, to investigate how these perturbations may impact sensitivity to T cell cytotoxicity.

We have developed a method for generating tumor cell lines that express a fragment of anti-CD3 antibody on the cell membrane.[9] These engineered cells provide 'signal 1' to T cells leading to T cell activation in an MHC- and antigen-independent manner, enabling T cells to be active in a redirected T cell-mediated cytotoxicity assay. This system allows for interrogation of intrinsic mechanisms of sensitivity/resistance to T cell-mediated cytotoxicity across a range of cell lines. In addition, we show that this versatile co-culture system can also be used to evaluate the activity of targeted therapies on both the tumor and T cell compartments across multiple donors.

Results

Optimizing CD8^{+} T cells for tumor cell cytotoxicity assays

To optimize our cytotoxicity assay, we first determined how *in vitro* restimulation conditions would impact the cytotoxic capacity and phenotype of CD8^{+} T cells. We used anti-CD3 redirected cytotoxicity against P815 mouse mastocytoma cells. These P815 cells express Fcγ-receptors, thus providing 'signal 1' and leading to CD8^{+}T cell activation and cytotoxicity. This assessment was used to guide subsequent experiments in the anti-CD3-expressing engineered tumor cell system used. Our co-culture assay is based on two rounds of anti-CD3/CD28 stimulation of isolated primary CD8^{+} T cells, which are then co-cultured with

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DiO labeled tumor cells at various time points. After which, staining with a LIVE/DEAD Violet viability dye allows for flow cytometry analysis of live DiO$^+$ Violet$^-$ tumor cells within the co-culture (Supplementary Fig. S1). Our data demonstrate that this second round of restimulation of CD8$^+$ T cells following in vitro expansion with anti-CD3/CD28 dynabeads, 5 days prior to co-culture in a cytotoxicity assay, enhanced effector function (Figure 1a). At the highest effector: target (E: T) ratio of 10:1 versus the control 0 E: T ratio, we observed approximately 30% P815 cytotoxicity in the presence of soluble anti-CD3, when compared to almost no effect with the isotype control. Comparatively, P815 cytotoxicity was increased to 45% in the co-culture at the 10:1 E: T ratio, following restimulation of the CD8$^+$ T cells (Figure 1a). This increased cytotoxicity was commensurate with an observed differentiation of the restimulated CD8$^+$ T cells

towards an effector phenotype (increased CD45RO expression, decreased CD45RA expression (Figure 1b,c), and decreased CCR7 expression (Figure 1d,e) when compared to pre-stimulation).

Using this expansion/reactivation protocol, we were able to generate sufficient populations of effectors T cells with robust cytotoxic activity for use in cytotoxicity assays.

Generation of anti-CD3 expressing tumor cell lines

Allogeneic T cell cytotoxicity assays are highly variable.[8] Therefore, TCR triggering through CD3 is an effective method for circumventing MHC/TCR alloreactivity and increasing the number of cytotoxic synapses between T cells and tumor cells. However, in contrast to P815 cells, most solid tumor cells lack Fc receptors and therefore TCR triggering at the synapse of T and
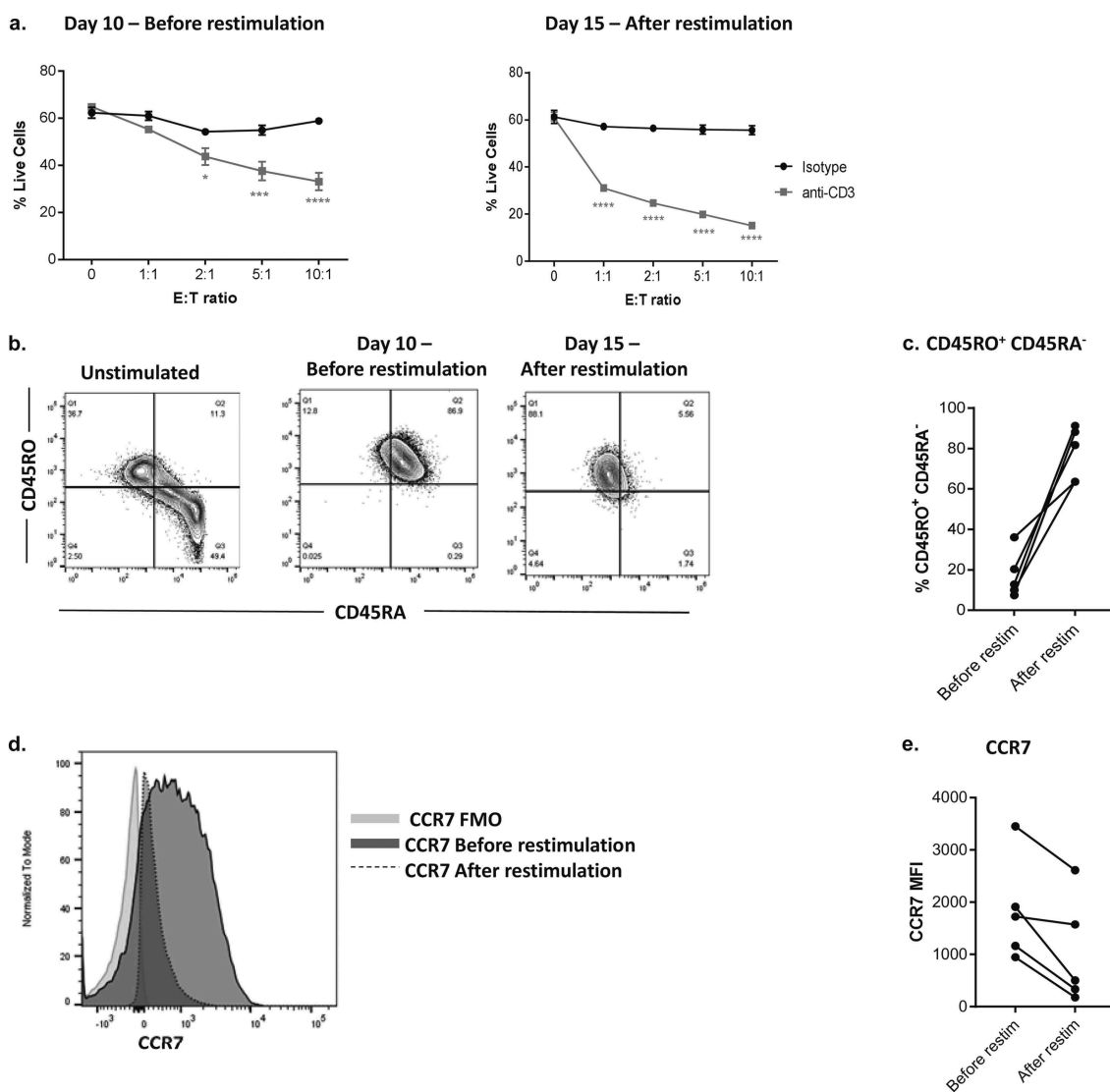

Figure 1. Optimizing CD8$^+$ T cells for tumor cell cytotoxicity assays.

a. Percentages of live P815 target cells in the presence of soluble anti-CD3 antibody or isotype control antibody in 4-h co-cultures with CD8$^+$ T cells before (left, day 10 after initial activation with anti-CD3/CD28 dynabeads) and after (right, day 15) a second round of stimulation with anti-CD3/CD28 dynabeads (performed on day 10). **b.** CD45RO and CD45RA expression by flow cytometry on naïve, unstimulated CD8$^+$ T cells, CD8$^+$ T cells on day 10 before and day 15 after restimulation **c.** Percentages of CD45RO$^+$CD45RA$^-$ CD8$^+$T cells across multiple donors, before and after restimulation by flow cytometry. **d.** Representative histogram showing CCR7 expression by flow cytometry in CD8$^+$ T cells before and after restimulation. **e.** MFI of CCR7 expression in CD8$^+$ T cells across multiple donors, before and after restimulation. Data is representative of two donors from three independent experiments (mean ± SEM). E: T ratio = effector: target ratio; MFI = Mean Fluorescence Intensity; FMO = fluorescence minus one control. *$P < 0.05$, and ***$P < 0.0005$ and ****$P < 0.0001$, significant difference of % live cells between isotype and anti-CD3 at each E: T ratio, assessed by two-way ANOVA and Sidak's multiple comparison test.
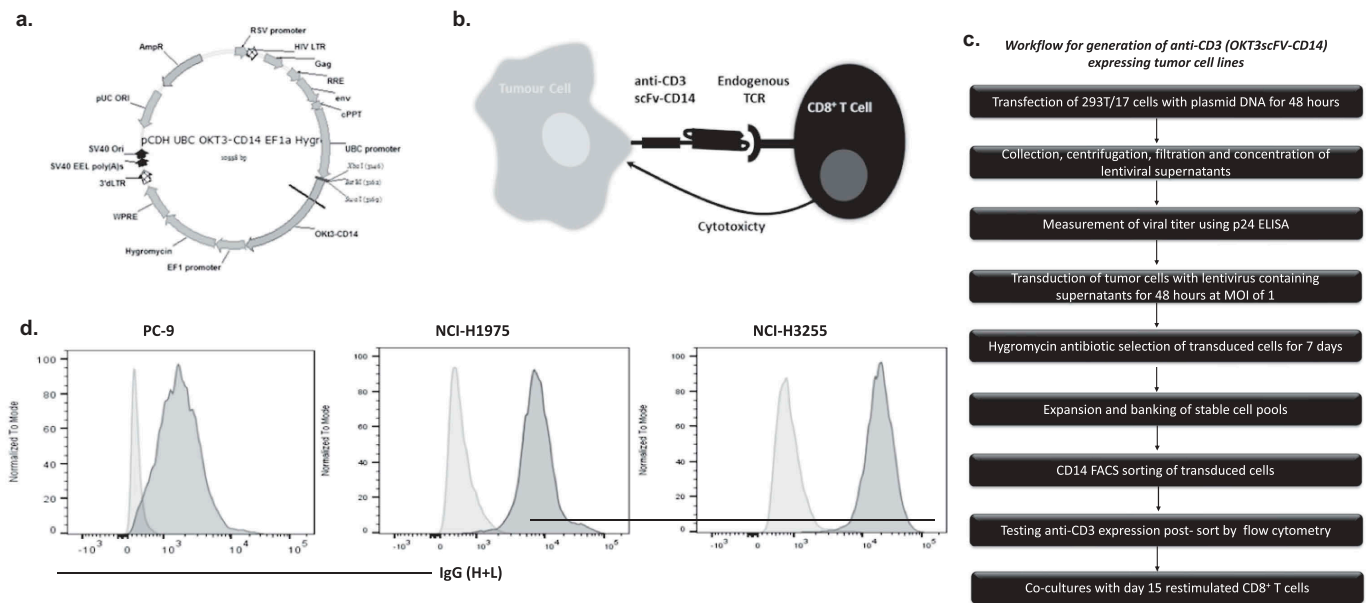
Figure 2. Generation of anti-CD3 expressing tumor cell lines.
a. Schematic of anti-CD3-scFV-CD14 expression construct design. **b.** Schematic of anti-CD3-scFV-CD14 expressing engineered tumor cells activating CD8^{+} T cells via the T cell receptor (TCR), leading to T cell-mediated cytotoxicity **c.** Workflow of experimental steps to generate stable anti-CD3-scFV-CD14 expressing tumor cell lines for use in co-culture assays with activated CD8^{+} T cells. **d.** Representative histograms showing anti-CD3 expression on parental (light grey histograms) and transduced (dark grey histograms) PC-9, NCI-H1975 and NCI-H3255 EGFRm NSCLC cell lines, as measured by flow cytometry after surface staining with anti-IgG. Data are representative of three independent experiments. MOI = multiplicity of infection; EGFRm = epidermal growth factor receptor mutant; NSCLC = non-small cell lung carcinoma.

tumor cells cannot be facilitated by crosslinking via addition of anti-CD3 antibody. Therefore, we generated lentiviral constructs encoding an anti-CD3 antibody fragment fused to the transmembrane domain of CD14[9] (Figure 2a). Three EGFR mutant (EGFRm) NSCLC cell lines; PC-9, NCI-H1975, and NCI-H3255, were transduced to express this scFV-CD3(OKT3)-CD14 construct to activate CD8^{+} T cells via their TCR and thus lead to cytotoxicity of the tumor cells (Figure 2b). The workflow for cell line transduction is provided in Figure 2c. Briefly, the anti-CD3 expressing tumor cells were transduced using viral supernatants from transfected 293T/17 cells. Multiplicity of infection (MOI) of both 1 and 10 were tested and based on sufficient transduction efficiency being achieved, cells transduced at an MOI of 1 were used for all experiments. Antibiotic selection was used to select for and maintain expression of the anti-CD3 scFv construct and transduced cells sorted by flow cytometry based on CD14 expression. Using an anti-IgG antibody (H + L), which binds to the heavy and light chains of the anti-CD3 scFV,[9] we examined the expression of cell surface anti-CD3 and found that all transduced cell lines expressed high levels of anti-CD3 relative to non-transduced parental controls (Figure 2d). Using this method, cell surface expression of anti-CD3 was routinely confirmed on the tumor cells lines.

Co-culture of CD8^{+} T cells with tumor cells expressing anti-CD3 results in T cell-mediated cytotoxicity

To validate the model, anti-CD3 expressing tumor cell lines were co-cultured with restimulated CD8^{+} effector T cells. T cell-mediated cytotoxicity against all three cell lines was evaluated at 4 and 18 h in comparison to P815 control cells, which were used as an indicator of T cell effector function due to their high susceptibility to T cell cytotoxicity as previously shown. Using our flow cytometry-based readout to analyse live tumor cells in these co-cultures, we observed that in contrast to control P815 cells (where CD3 stimulation is regulated by soluble anti-CD3 antibody), the window of T cell-mediated cytotoxicity in the anti-CD3 expressing NSCLC cell lines was small at 4 h (roughly, 3% in PC-9, 11% in NCI-H1975 and 17% in NCI-H3255) (as calculated by differences in cytotoxicity at 0 to 10:1 E: T ratio) (Figure 3a). However, by 18 h, the magnitude of T cell-mediated cytotoxicity was greater in both the P815 cells and in PC-9, NCI-H1975 and NCI-H3255 EGFRm cell lines (Figure 3b). As expected, the level of cytotoxicity was proportional to the E: T ratio. In these 18h co-cultures, the range of tumor cell death varied from 30% to 70% at the lowest E: T ratio (1:1) to 80–100% at the highest E: T ratio (10:1). We also show that the observed trends in cytotoxicity across all three EGFRm cell lines in 18h co-cultures, is replicated across multiple donors and experiments tested, with some variability depending on donor responses (Supplementary Fig. S2). Each cell line displayed slight variation in their intrinsic sensitivity to cytotoxicity in this assay. To explore this further, we also evaluated T cell function following co-culture with the different tumor cell lines, by measuring granzyme B and PD-1 expression by intracellular staining of CD8^{+} T cells and flow cytometry (Figure 3c,d). Granzyme B production (Figure 3c) and PD-1 (Figure 3d) expression were induced in CD8^{+} T cells by all three tumor cell lines, compared to CD8^{+} T cells alone, although significance was only observed in PC-9 and NCI-H1975 cells lines at all E: T ratios. However, each cell line induced differing total levels of granzyme B and PD-1, with T cells co-cultured with PC-9 tumor cells expressing the highest level of granzyme B (Figure 3c), while T cells co-culture with NCI-H1975 tumor cells expressed the highest level of PD-1 (Figure 3d).
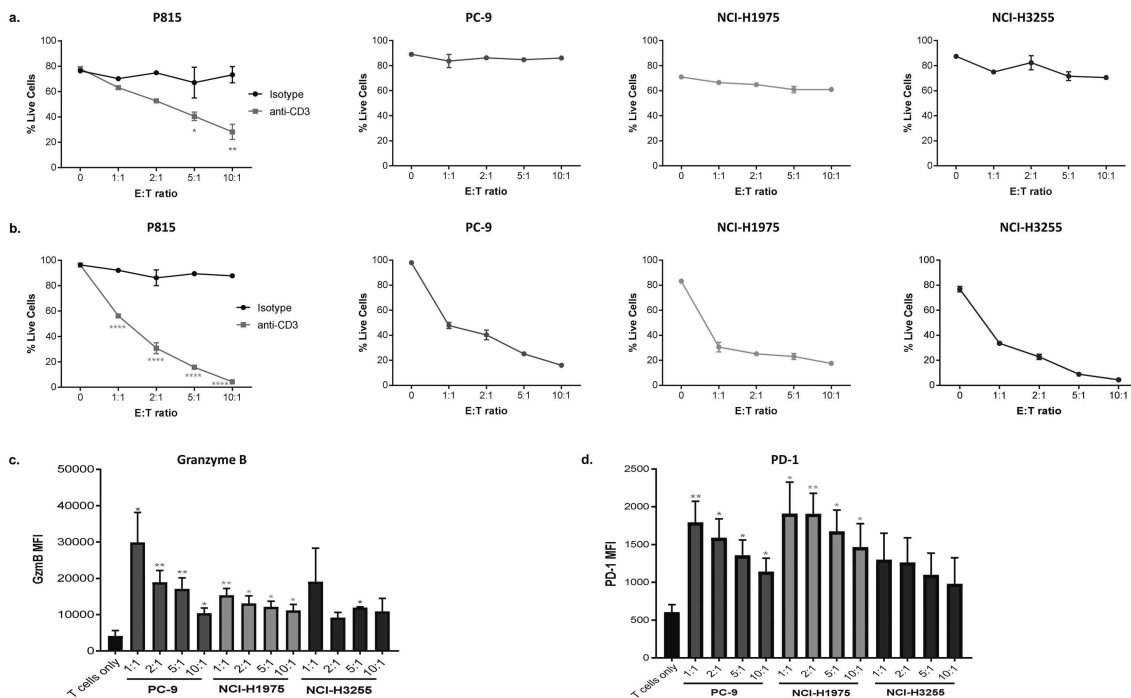
Figure 3. Co-culture of CD8$^+$ T cells with tumor cells expressing anti-CD3 results in target cell T cell-mediated cytotoxicity.

a. Percentages of live P815 cells (in the presence of soluble anti-CD3 antibody or isotype control antibody) and PC-9, NCI-H1975 and NCI-H3255 anti-CD3 engineered EGFRm cell lines in co-cultures with restimulated CD8$^+$ T cells for 4 h. **b.** Percentages of live P815 cells and anti-CD3 expressing EGFRm cell lines in co-cultures with restimulated CD8$^+$ T cells for 18 h. MFI of **c.** Granzyme B expression and **d.** PD-1 expression in CD8$^+$ T cells alone and in co-cultures with anti-CD3 expressing PC-9, NCI-H1975 and NCI-H3255 EGFRm cell lines. Data are representative of two donors from three independent experiments (mean ± SEM). EGFRm = epidermal growth factor receptor mutant; GzmB = granzyme B; MFI = Mean Fluorescence Intensity. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$ and ****$P < 0.0001$, significant difference of % live cells between isotype and anti-CD3 at each E: T ratio (Figure 3a,b) and MFI of granzyme B and PD-1 between T cells only and at each E: T ratio (Figure 3c,d), assessed by two-way ANOVA with Sidak's multiple comparison test and unpaired parametric two-tailed t-test, respectively.

Expression levels of anti-CD3 influence the extent of tumor cell cytotoxicity by T cells

As expected, the level of anti-CD3 expression influences the magnitude of T cell-mediated cytotoxicity. Using a flow cytometric gating system, we were able to demonstrate this by gating on cells with high, medium and low expression using anti-IgG (H + L) staining (Figure 4a). Using, PC-9 cells (transduced with anti-CD3), we show that expression of anti-CD3 correlates with cytotoxicity, especially at higher E: T ratios (Figure 4b). Therefore, this gating can be applied to potentially explore the impact of TCR signal agonism on cytotoxic responses. Moreover, to circumvent this issue when comparing across cell lines with different levels of anti-CD3 expression (Supplementary Fig. S3A), this gating strategy can be specifically applied (Supplementary Fig. S3B) to select cells with similar levels of anti-CD3 expression (Supplementary Fig. S3C). This method can be used to control for variability in transduction efficiencies, thus permitting the analysis of a population of cells with highly overlapping expression levels of anti-CD3 and allowing for direct comparisons of relative cytotoxicity across multiple cell lines (Supplementary Fig. S3D).

Measurements of IFN-ɣ and early tumor cell apoptotic events provide additional sensitive pharmacodynamic endpoints in this assay system

On recognition of peptide-MHC complexes on target cells, CD8$^+$ T cells trigger programmed cell death of target cells.[10] Therefore, we wanted to characterize the mode of cell death in

the anti-CD3-transduced tumor cells beyond evaluating overall viability in co-cultures. We examined cell death in our co-cultures using both direct DiO labelling of mock (cell lines transduced with empty vector controls) and anti-CD3 transduced tumor cells (Figure 5a) and using annexin-V and 7-amino actinomycin D (7-AAD) staining of co-cultures with anti-CD3 expressing cells only, as a readout to evaluate apoptosis (Figure 5b). We found that anti-CD3 transduced tumor cells undergo significantly more T cell-mediated cytotoxicity than their empty vector mock control counterparts (Figure 5a) and that there is an induction of early apoptotic tumor cells (annexin-V$^+$ 7-AAD$^-$) in the co-cultures with the anti-CD3 expressing NSCLC cell lines (Figure 5b). Moreover, measurement of IFN-ɣ in the co-culture supernatants of mock and anti-CD3 tumor cells also correlated with the level of tumor cell cytotoxicity and E: T ratios (Figure 5c) and was sufficiently sensitive to be a pharmacodynamic readout of T cell activity.

Investigating the impact of targeted therapies on T cell-mediated cytotoxicity using the CD3-expressing tumor cell co-culture system

Evaluating the potential impact of targeted therapies on both tumor and T cell responses is becoming increasingly important as immunotherapy becomes part of the standard of care for many cancer types. Targeted therapies may enhance or have antagonistic effects on T cell-mediated cytotoxicity. Moreover,
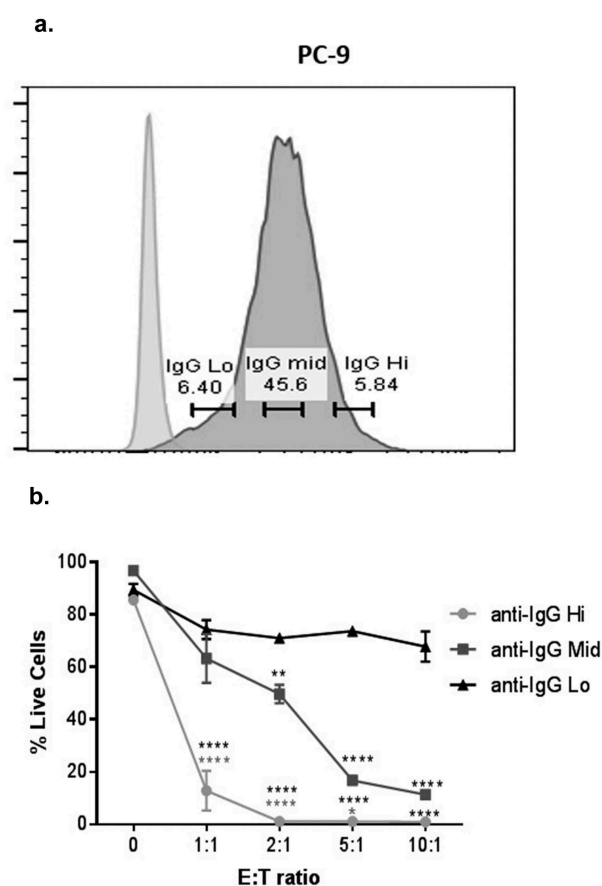
Figure 4. Expression levels of anti-CD3 influence the extent of tumor cell cytotoxicity by T cells.

a. Representative histograms showing IgG hi, mid and lo gates on EGFRm PC-9 anti-CD3 expressing cells (dark grey histogram) compared to FMO controls (light grey histogram), using flow cytometry staining with anti-IgG. **b.** Percentages of live PC-9 anti-CD3 transduced cell lines when gating on IgG Hi, mid and lo tumor cell expression after 18h co-cultures with restimulated CD8+ T cells. Data are representative of two donors from three independent experiments (mean ± SEM). EGFRm = epidermal growth factor receptor mutant; FMO = fluorescence minus one control. ******$P < 0.01$ and ********$P < 0.0001$, significant difference of % live cells between anti-IgG Mid vs anti-IgG lo anti-IgG Hi vs anti-IgG lo at each E: T ratio, assessed by two-way ANOVA and Sidak's multiple comparison test.

evaluating how combinations of distinct immunotherapies may converge on cytotoxic potential against tumor cells is poorly understood.[11] Therefore, using the PC-9 EGFRm anti-CD3 expressing NSCLC cell line as a model, we next characterized the effects of EGFR tyrosine kinase inhibitors (TKIs) on T cell cytotoxic potential. PC-9 cells harbour an EGFR activating mutation due to a deletion in exon 19[12,13] and are therefore sensitive to first and third generation EGFR TKIs, gefitinib and osimertinib, respectively.[14] Pre-treatment of PC-9 cells with gefitinib and osimertinib, at clinically relevant doses and maintenance of these concentrations in co-cultures, resulted in an increase in the amount of tumor cell death observed in co-cultures with both drugs, compared to DMSO control (Figure 6a). This increase in tumor cell death was mirrored in the no T cell controls (0 E: T ratio) and increased the amount of tumor cell death evaluated in combination with effector CD8+ T cells (Figure 6a). These observations were also confirmed using the anti-CD3 expressing NCI-H3255 EGFR mutant NSCLC cell line (Supplementary Fig. S4). These findings suggest that inhibition of EGFR signaling, whilst capable of reducing the

survival of EGFRm NSCLC cells, does not negatively influence T cell-mediated cytotoxicity.

Adenosine has been shown to have suppressive effects on T cell function and is a component of some tumor microenvironments.[15,16] The addition of SCH58261; a selective inhibitor of A2aR receptor,[16] was not found to directly impact tumor cell viability (0 E: T ratio), but was found to significantly enhance T cell-mediated cytotoxicity against anti-CD3 transduced PC-9 cells in the presence of AMP, when compared to DMSO control, across all E: T ratios tested (Figure 6b). Moreover, we show this increase in tumor cell death can be consistently observed across donors (Supplementary Fig. S5). As such, these data suggest that targeting the adenosine pathway can directly modulate CD8+ T cell cytotoxic function.

To further characterize the assay, we next investigated the effects of dasatinib, a potent inhibitor of the Src family kinases including Lck which is critical for mediating T-cell signaling downstream of the TCR.[17,18] Our assay demonstrated a significant reduction in T cell-mediated cytotoxicity against anti-CD3 expressing PC-9 cells in the presence of 12.5 nM dasatinib compared to DMSO control and revealed that dasatinib had no effect on tumor cells alone, in the absence of T cells (0 E: T ratio) (Figure 6c). Furthermore, a dose-response of dasatinib from 0 to 100 nM in co-cultures with anti-CD3 expressing PC-9 cells showed that concentrations of 25, 50 and 100 nM dasatinib further significantly reduced T cell-mediated cytotoxicity of PC-9 cells (Supplementary Fig. S6). These findings confirm that modulation of TCR signaling could be measured through the magnitude T cell-mediated tumor cell cytotoxicity in the co-culture assay. Lastly, we investigated the effects of these targeted therapies on CD8+ T cell viability to exclude direct effects on T cells. We found no significant difference in the viability of CD8+ T cells when treated with gefitinib (320 nM), osimertinib (160 nM) (Supplementary Fig. S7A), SCH58261 (10 μM) (Supplementary Fig. S7B) and dasatinib (12.5 nM) (Supplementary Fig. S7C), at the same concentrations and time points used in co-culture assays.

Together these data demonstrate that this novel co-culture system can be easily applied to explore, how targeted therapies may impact both the T cell and tumor cell compartments across multiple donors, without the intrinsic variabilities normally observed with allogeneic responses.

Discussion

Understanding how CD8+ T cell anti-tumor immune responses are conditioned by specific features of tumors such as their tissue of origin and genotypes could aid in the development of successful immunotherapy treatments. Within the tumor microenvironment, this complex interaction is influenced by the presence of a number of inhibitory and costimulatory surface molecules on tumor cells and cytokines that can affect CD8+ T cell cytotoxic function.[19] In the system described here, we propose an *in vitro* approach to evaluate tumor cell sensitivity to CD8+ T cell-mediated cytotoxicity via their expression of anti-CD3 (OKT3). The ability of these engineered tumor cells to provide 'signal 1' to CD8+ T cells, thereby directly activating them, bypasses the need for TCR recognition of specific MHC-
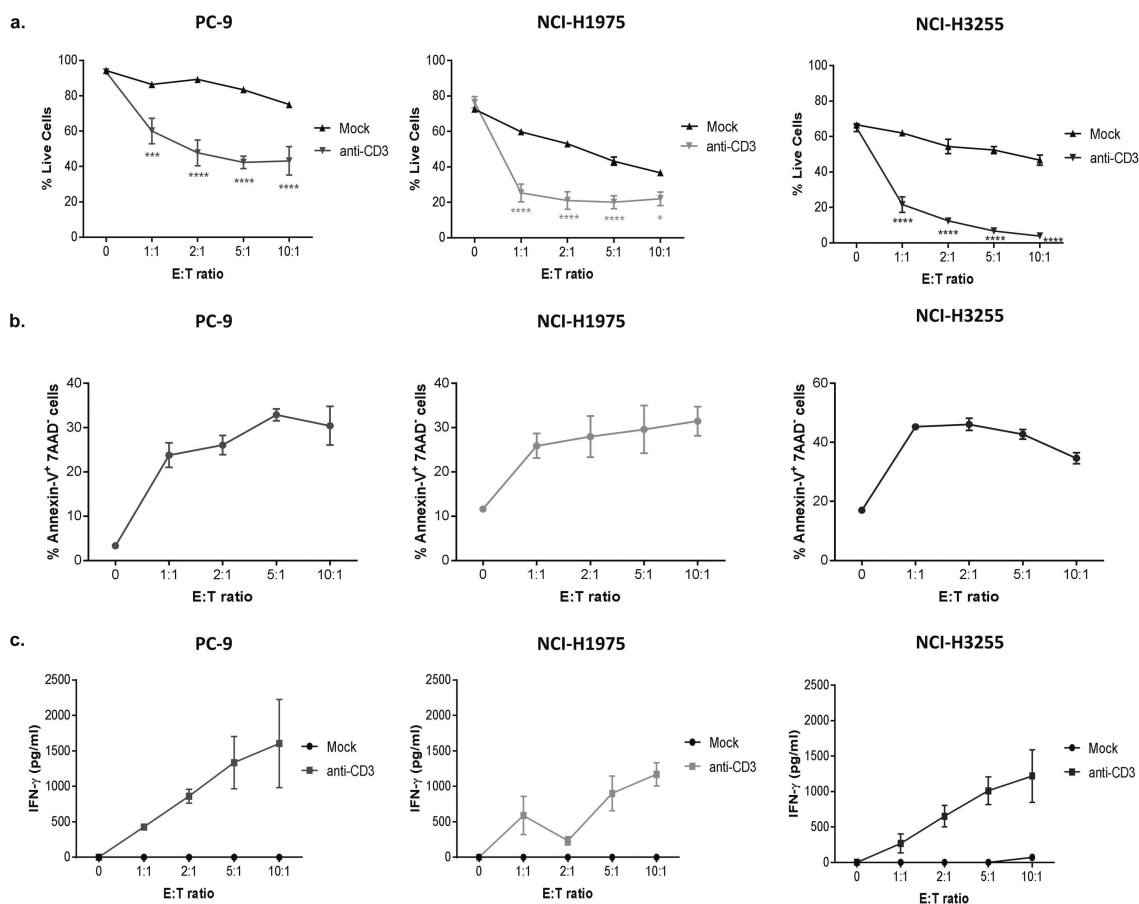
Figure 5. Measurements of IFN-γ and early tumor cell apoptotic events provide additional sensitive pharmacodynamic endpoints in this assay system.
a. Percentages of live mock control cells versus anti-CD3 expressing PC-9, NCI-H1975 and NCI-H3255 EGFRm cell lines after 18h co-cultures with restimulated CD8^{+} T cells **b.** Percentages of annexin-V^{+} 7-AAD^{−} (apoptotic) anti-CD3 expressing PC-9, NCI-H1975 and NCI-H3255 EGFRm cell lines after 18h co-cultures with CD8^{+} T cells. **c.** Levels of IFN-γ production in supernatants from **a.** after 18h co-cultures of CD8^{+} T cells with mock and anti-CD3 expressing PC-9, NCI-H1975 and NCI-H3255 EGFRm tumor cell lines. Data are representative of two donors from three independent experiments (mean ± SEM). EGFRm = epidermal growth factor receptor mutant. *$P < 0.05$, ***$P < 0.0005$ and ****$P < 0.0001$, significant difference of % live cells between mock and anti-CD3 at each E: T ratio, assessed by two-way ANOVA and Sidak's multiple comparison test.
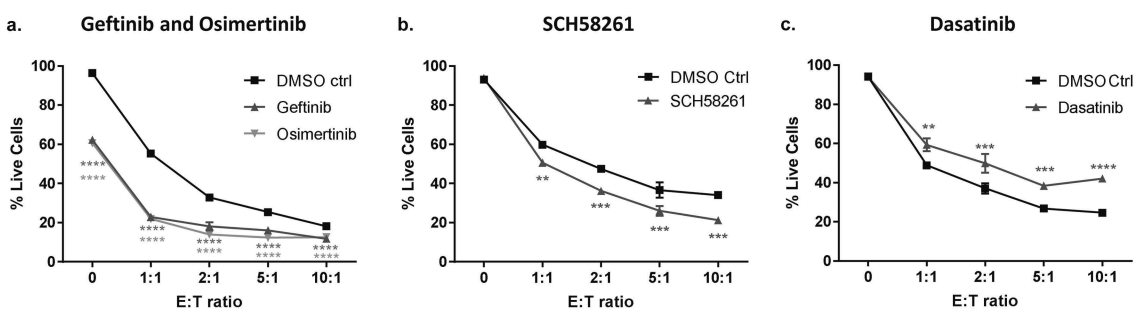

Figure 6. Investigating the impact of targeted therapies on T cell-mediated cytotoxicity using the anti-CD3-expressing tumor cell co-culture system.
Percentages of live PC-9 EGFRm anti-CD3 expressing cells after 18-h co-culture with restimulated CD8^{+} T cells **a.** with PC-9 pre-treatment with 360 nM Geftinib and 160 nM Osimertinib EGFR TKIs **b.** in the presence of 10 μM SCH68261 and **c.** in the presence of 12.5 nM Dasatinib, compared to DMSO controls. Data are representative of two donors from two independent experiments (mean ± SEM), with two different donors used in each drug treatment experiment. EGFRm = epidermal growth factor receptor mutant; EGFR TKIs = epidermal growth factor receptor tyrosine kinase inhibitors; DMSO ctrl = DMSO control. **$P < 0.01$, and ***$P < 0.0005$ and ****$P < 0.0001$, significant difference of % live cells between drug treatments and DMSO ctrl at each E: T ratio, assessed by two-way ANOVA and Sidak's multiple comparison test.

peptide complexes on tumor cells, whilst still allowing for the contributions of endogenous mechanisms of sensitivity and resistance to be assessed. Thus far, assays that allow for simultaneous assessment of CD8^{+} T cell-mediated cytotoxicity of different cell lines across common donors are limited due to HLA restriction and the variability associated with allogeneic responses.[5,6] Although not antigen-specific, our assay presents a novel approach to investigating tumor escape strategies between and within various cancer types, in anti-CD3 engineered co-culture assays. In this paper, we specifically examined

EGFRm NSCLC cell types and found a degree of variability in their sensitivities to T cell-mediated cytotoxicity. The determinants of these variations to T cell-mediated cytotoxicity will be investigated in future studies. Such insight could lead to the development of more personalized therapies in which the selection of drug treatments could potentially be based on tumor genetics and predicted anti-tumor immune responses, using assays such as the one described here.

Historically, the "gold standard" for measuring cytotoxicity has been assays based on chromium (Cr[51]) released by the lysed target cells.[7] This method involves the use of radioactive materials and the measurement of target cell death and has low sensitivity in assays of cultures longer than 4–8h due to the spontaneous release of Cr[51.20,21] Alternatively, assays that measure the release of endogenous enzymes[22] and cytokines[23] from co-culture supernatants indirectly measure cytotoxicity and may not adequately compensate for the contribution of effector cells in the readout.[6,7] Our method relies on a flow cytometry-based readout allowing for the evaluation of direct T cell-mediated cytotoxicity by measuring tumor cell death. Moreover, the method supports the analysis of the mechanism of cell death, e.g. via measurement of annexin-V and 7AAD on tumor cells and can provide insight into T cell viability and phenotype following co-culture with target cells.

The generation of membrane-anchored anti-CD3 scFV expressing cells has been previously described to create immunosomes[24] and stimulator cells,[9] which both activate human T cells. However, this is the first description of this approach to directly engineer tumor cells to explore the cytotoxic potential of CD8^{+} T cells. We have shown that this protocol is amenable to a variety of adherent tumor lines and as such, can facilitate comparisons of tumor cell sensitivity across multiple tumor cells. Our data show that T cell-mediated cytotoxicity of tumor cells is comparable to that of P815 cells which express Fc receptors that allow TCR crosslinking in the presence of soluble anti-CD3 (Figure 3b). Whilst there remains some donor to donor variability in the degree of cytotoxicity observed, this is reduced when compared to assays that rely upon alloreactivity.[8,25] However, it is evident that the expression of anti-CD3 on tumor cell lines leads to a larger cytotoxicity window compared to an allogeneic reaction (Figure 5 – anti-CD3 vs. mock controls). This novel method provides a larger therapeutic window to evaluate pharmacodynamic responses and increases assay throughput by reducing the number of T cells required to elicit robust cytotoxicity, both of which are limitations with standard allogeneic cytotoxicity assays. Moreover, our data demonstrate that the T cell-mediated cytotoxicity observed in this assay is specifically driven via anti-CD3 expression as empty vector transduced (mock controls) cells undergo very little cell death via allorecognition in this assay and failed to induce IFN-γ production by CD8^{+} T cells when compared to co-cultures with anti-CD3 expressing tumor cells.

To optimize and standardize the methods for preparation of the CD8^{+} T cells used in the assay, we have characterized both their function and phenotype during pre-activation as well as during co-cultures. We have shown that a second round of stimulation of T cells with anti-CD3/CD28 leads to increased expression of CD45RO and loss of CD45RA and

CCR7, resulting in increased effector function indicated by T cell-mediated cytotoxicity of P815 target cells (Figure 1a). Within co-cultures, CD8^{+} T cell cytotoxic function was evident not only by the cell death of tumor cells but also the significant production of granzyme B, in response to anti-CD3 stimulation by tumor cells. Previous studies have shown that on TCR crosslinking, stored granzyme B is released and there is *de novo* synthesis of lytic proteins that refills these granules, which are in turn secreted[26] and might therefore explain our results. PD-1 has important immunoregulatory roles in limiting effective T cell responses.[27] Traditionally, PD-1 has been noted as one of the hallmarks of T cell exhaustion. However, a broader view of PD-1 has highlighted its importance as firstly, being a marker of T cell activation and secondly, its expression correlating with TCR signaling strength.[28] As such, the induction of PD-1 expression in CD8^{+} T cells by anti-CD3 expressing tumor cells is expected and further shows the physiological relevance of our system.

Previously published studies demonstrate that the level of TCR engagement, e.g. by increased level of MHC/TCR clustering, can influence the relative susceptibility of a target cell to T cell-mediated cytotoxicity.[29,30] In our assay system, we describe a simple gating strategy to enable measurement of the anti-CD3 expression levels on transduced cells in co-cultures and show that this correlates with the amount of T cell-mediated cytotoxicity observed. This is particularly useful in manipulating the amount of cytotoxicity observed in a single cell line, depending on the intended application of the assay. One caveat of this novel method is differing transduction efficiencies of tumor cells, which can result in varying levels of anti-CD3 expression across different cell lines. Therefore, this gating strategy allows cytotoxicity readouts to be normalized via anti-CD3 expression across cell lines.

Targeted and immunotherapies have been extensively investigated for their effects on cancer cells and in some cases, immune cells. However, as it relates to CD8^{+} T cells, most of these studies have evaluated their effects on assays such as proliferation and cytokine production *in vitro and in vivo*, which indirectly reflect cytotoxic function.[31] We have shown that our system directly allows the investigation of therapies of interest on T cell cytolytic activity. We first showed that EGFR TKIs result in an increase in target cell death in PC-9 cells, which appears to be as a consequence of their direct cytotoxic effect on the target cell and correlates with the well-reported sensitivities of this cell line to EGFR TKI based on its genotype.[14] These results appear to be additive in PC-9 and in a second EGFR mutant NSCLC cell line, NCI-H3255 (also characterised to be sensitive to both gefitinib and osimertinib)[14] at lower E: T ratios, suggesting that the effects of EGFR TKI treatment are diminished when T cell-mediated cytotoxicity is increased in the presence of higher numbers of CD8^{+} T cells. The reason for this is unclear but there may be a maximum threshold of cytotoxicity measurable in this assay. We also observed that SCH58261, an inhibitor of adenosine A2a receptor (A2aR), increases T cell-mediated cytotoxicity of target cells. Furthermore, this increase of cytotoxicity with SCH58261 is consistent regardless of donor variability, and overall suggests that adenosine could in fact influence T cell effector lytic function.[32] We also

showed that the Lck inhibitor, dasatinib, decreased the amount of T cell-mediated cytotoxicity observed, showing that our assay is amenable to both enhancing and suppressive effects.

Overall, we have detailed a system that allows for engineering tumor cells to express anti-CD3 to measure their sensitivity to T cell-mediated cytotoxicity while also simultaneously phenotyping CD8^{+} T cells and their responses, in an *in vitro* co-culture assay. This system is highly adaptable to test different tumor cells, is reproducible across different donor T cells and is both robust and sensitive permitting the evaluation of novel drug combinations and interrogation of tumor intrinsic mechanisms that may influence susceptibility to T cell-mediated cytotoxicity.

Materials and methods

Generation of expression constructs encoding membrane-bound anti-CD3 single chain fragments

cDNA derived from hybridoma cells producing the OKT3-CD14 fusion protein (anti-CD3), was subjected to PCR amplification using primer pairs 5′-TTCGAAGCCACCATGCCT ATGGGCTC-3′ and 5′-GGATCCTCAGGCGAAGCCTCT AG-3′ designed with BstB1 sites on the 3′ end and BamH1 5′ end, digested and cloned into pCDH-UbC-MCS-EF1a-Hygro to generate the lentiviral plasmid pCDH-UbC-CD14-OKT3-EF1a-Hygro.

Cell culture and generation of anti-CD3 expressing tumor cell lines – lentiviral transfection and transduction

For lentiviral transfection, 293T/17 packaging cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% heat-inactivated/gamma irradiated fetal bovine serum (FBS) (Invitrogen) and 1mM MEM non-essential amino acids (Gibco) at 37°C and 5% CO2. At 70–90% confluence, 293T/17 cells were lentivirally transfected with pCDH-UbC-CD14-OKT3-EF1a-Hygro expression plasmid or a pCDH-UBC-EF1a-Hygro empty plasmid (for mock controls) and a master mix of packaging plasmids (pVSV-G, pPACK-HI-GAG and Rev-pPACK-HI-REV) using lipofectamine and Opti-MEM (Gibco). Supernatant containing lentivirus was collected 48 h later, filtered through a 0.2 μm filter (EMD Millipore) and aliquots stored at −80°C for transduction of tumor cells. A Lenti-X™ p24 Rapid Titer Kit (Clontech) was used to determine lentiviral titers of collected supernatants. All tumor cells were maintained in RPMI Medium 1640 – GlutaMAX™-I (Gibco) supplemented with 10% FBS, at 37°C and 5% CO2. PC-9, NCI-H1975 and NCI-H3255 EGFRm tumor cells were grown to 80% confluence and were retrovirally transduced using lentiviral supernatants at a multiplicity of infection (MOI) of 1 and 10, based on p24 ELISA results, in the presence of polybrene for 48 h. Positively expressing cells were selected for using hygromycin B (Invitrogen) for 1 week, cells were then maintained in hygromycin (30 μg/ml for PC-9 and NCI-H3255; 60 μg/ml for NCI-H1975), passaged and bulked up for all experiments. Anti-CD3 transgene expression was confirmed in cells transduced at a MOI of 1 and 10 by flow

cytometry staining and all future experiments were conducted with cells transduced with an MOI of 1.

Sorting of anti-CD3 expressing tumor cell lines

Expression of CD14 membrane-bound anti-CD3 OKT3 scFV on transduced cells was detected using either an Alexa Fluor® 647 AffiniPure Fab Fragment Goat anti-Mouse IgG (H + L) antibody (Jackson Immunoresearch) or human CD14 PE-conjugated antibody (R&D Systems). For sorting of transduced cells, PC-9 NCI-H1975 and NCI-H3255 anti-CD3-expressing cell lines were stained with human CD14 PE-conjugated antibody (R&D Systems) and parental (non-anti-CD3 expressing) cells were used as negative controls. PC-9, NCI-H3255 and NCI-H1975 high expressing CD14 cells, with overlapping expression levels, were gated for and sorted on a FACS Aria sorter. Sorted cells were cultured in the presence of hygromycin as before, passaged and bulked up for all experiments.

CD8^{+} T cell isolation, activation, and expansion

PBMCs were isolated from blood from healthy volunteer donors (National Health Service (NHS) blood and transplant unit at Addenbrooke's hospital) using density centrifugation with Ficoll-Paque Plus (GE Healthcare). CD8^{+} T cells were purified from PBMC using the EasySep™ Human CD8^{+} T Cell Enrichment Kit (Stem Cell Technologies) and a RoboSep (Stem Cell Technologies), according to the manufacturer's instructions. Isolated CD8^{+} T cells were activated with CD3/CD28 dynabeads (Thermo Scientific) at a 1:1 ratio, cultured in complete culture assay medium (DMEM- GlutaMAX™-I, 10% FBS) supplemented with 1% penicillin-streptomycin and 100 U/ml IL-2 (Peprotech) and maintained at a density of 1×10^6 cells/ml, with medium changed every 2–3 days. On day 10, CD8^{+} T cells were re-stimulated with CD3/CD28 dynabeads as above with medium changes every 2–3 days.

CD8^{+} T cell cytotoxic assay

One day prior to co-culture assays (day 14 of CD8^{+} T cell activation), P815, PC-9, NCI-H1975, and NCI-H3255 tumor cells were labeled with Vybrant DiO Cell-Labelling Solution (Thermo Fisher Scientific) at a 1:200 dilution for 8 min, according to the manufacturer's instructions. Cells were washed, resuspended at 2×10^5 cells/ml in culture assay medium and 50 μl/well (10,000 cells) plated in 96 well-U-bottom plates. Plates were centrifuged and incubated overnight at 37°C and 5% CO2. On day 15 of T-cell activation, CD8^{+} T cells were harvested, dynabeads removed magnetically and cells were resuspended at 1×10^6 cells/ml in culture assay medium. For all co-cultures, CD8^{+} T cells were added to respective wells of the U-bottom plate containing DiO-labelled tumor cells at 1:1, 2:1, 5:1 and 10:1 effector: target (E: T) ratios. Plates were centrifuged and incubated for either 4 or 18 h. After incubation, supernatants were removed and stored at −80°C, wells were washed with PBS and cells were detached with accutase and returned to the incubator for 10 min. Cells were then transferred to a 96-well V-bottom plate and centrifuged. Harvested cells were stained with a LIVE/DEAD Fixable

Violet Dead Cell Stain Kit (Thermo Scientific) at a dilution of 1:2500 in PBS for 10 min at room temperature. Cells were then washed, fixed in 3.7% formaldehyde and data acquisition carried out using a BD FACS Canto II or BD LSR Fortessa to analyze Violet$^+$ DiO $^-$ (live tumor cells). In some experiments, P815 mouse mastocytoma cells were used as controls to control for T cell-mediated cytotoxicity capacity. P815 cells were DiO labeled and plated as was done for tumor cells and were pre-treated with 0.5 μg/ml anti-CD3 (OKT3 clone) for 1 min (eBioscience) or an isotype-matched control, before the addition of CD8$^+$ T cells at their respective ratios. Where indicated, staining with an Alexa Fluor® 647 AffiniPure Fab Fragment Goat Anti-Mouse IgG (H + L) antibody was also done in experiments to gate on cells with similar anti-CD3 (OKT3) scFV expression levels[9] (Jackson ImmunoResearch). FITC Annexin-V Apoptosis Detection Kit I (BD Biosciences) was used in experiments to analyze apoptotic cell death in co-cultures. For co-culture experiments in the presence of EGFR tyrosine kinase inhibitors (TKIs), anti-CD3 tumor cells were pre-treated with 320 nM Gefitinib and 160 nM Osimertinib after being DiO-labelled, 24 h before the addition of CD8$^+$ T cells for 18 h. SCH58261 was added at a final concentration of 10 μM at the same time as the addition of the CD8$^+$ T cells, in the presence of 100 μM adenosine monophosphate (AMP) (Sigma Aldrich). Experiments using dasatinib (AstraZeneca in-house supply) were similarly carried out using a 12.5 nM concentration (as described by[17]) added at the same time as the CD8$^+$ T cells. Dasatinib dose-response experiments were similarly repeated using concentrations indicated. All drug concentrations were maintained throughout the 18h co-cultures. Flow cytometric analysis was performed using a BD Fortessa. Data were analyzed using FlowJo (Tree Star) as shown in Figure S1B.

T cell phenotyping

T cells were phenotyped by flow cytometry staining before (Day 0 – naïve and Day 10–10 days after 1st round of stimulation) and after restimulation (Day 15), as well as in co-cultures with tumor cells. Briefly, CD8$^+$ T cells were stained with LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Thermo Scientific), followed by surface staining at 4 ^{o}C with: – anti-CD8 AF647, anti-CD8 PerCPCy5.5, anti-CD45RO Alexa Fluor 488, anti-CD 45RA BV605, anti-CCR7-PE, anti-PD-1 PerCpCy5.5 (all from Biolegend) antibodies. Staining of CCR7 was performed at 37 ^{o}C for 30 min. Intracellular granzyme B staining was performed with anti-Granzyme B-AF647 antibody (Biolegend), after permeabilization with Fixation/Permeabilization Solution Kit (BD Biosciences). Flow cytometric analysis was performed using a BD Fortessa. Data were analyzed using FlowJo (Tree Star).

IFN-γ measurement

Supernatants from co-culture assays were stored at −80°C, prior to analysis for IFN-γ production using the Human IFN-γ DuoSet ELISA (R&D systems), according to the manufacturer's instructions. Plates were washed using a BioTek EL406 microplate washer dispenser and read using an Envision L plate reader (PerkinElmer).

Statistics

Two-way ANOVA and t-test were used to assess significance using PRISM software. The P values are represented as ****$P < 0.0001$ ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. The data are presented as mean ± standard error of the mean (SEM) or mean ± standard (SD) where indicated.

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No potential conflicts of interest were disclosed.

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