Cancer-cell stiffening via cholesterol depletion enhances adoptive T-cell immunotherapy

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Malignant transformation and tumour progression are associated with cancer-cell softening. Yet how the biomechanics of cancer cells affects T-cell-mediated cytotoxicity and thus the outcomes of adoptive T-cell immunotherapies is unknown. Here we show that T-cell-mediated cancer-cell killing is hampered for cortically soft cancer cells, which have plasma membranes enriched in cholesterol, and that cancer-cell stiffening via cholesterol depletion augments T-cell cytotoxicity and enhances the efficacy of adoptive T-cell therapy against solid tumours in mice. We also show that the enhanced cytotoxicity against stiffened cancer cells is mediated by augmented T-cell forces arising from an increased accumulation of filamentous actin at the immunological synapse, and that cancer-cell stiffening has negligible influence on: T-cell-receptor signalling, production of cytolytic proteins such as granzyme B, secretion of interferon gamma and tumour necrosis factor alpha, and Fas-receptor–Fas-ligand interactions. Our findings reveal a mechanical immune checkpoint that could be targeted therapeutically to improve the effectiveness of cancer immunotherapies.

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Results

The plasma membrane of cancer cells is enriched with cholesterol. We first explored how cancer cells soften their cortical structure. It has been reported that depleting cholesterol from the plasma membrane in endothelial cells increases cell cortical stiffness. These studies suggest that high membrane cholesterol level correlates with decreased cell stiffness. We therefore measured the cholesterol levels in murine and human tumour tissues as well as cells isolated from those tissues by staining with Filipin III, a fluorescent dye that binds specifically to cholesterol. Interestingly, in the B16F10 murine melanoma model, histological analysis showed that tumour tissues exhibited a substantially higher cholesterol level compared with the paired healthy tissues (Fig. 1a). In line with the results of histological studies, B16F10 tumours showed a 4.0- and
Fig. 1 | Cholesterol is enriched in the plasma membrane of cancer cells. a, B16F10 tumour tissues (indicated with dash lines) and the adjacent normal tissue were stained with H&E and Filipin III (shown in blue colour). Scale bar, 500 μm. b, Cholesterol levels in B16F10 tumour tissues and the adjacent skin and muscle tissues (n=3). c–d, Membrane cholesterol levels of tumour-infiltrating leucocytes (CD45.2+ and cancer cells (tdTomato+) in 4T1-Fluc-tdTomato tumour (c) and murine T lymphoma cells (EG7-OVA) and normal murine T cells (d) determined by Filipin III staining (n=3). The displayed MFI values were normalized by the forward scatter area (FSC-A) of corresponding samples. e, Relative intracellular and plasma membrane levels of cholesterol (normalized to that of native cells) in murine B16F10 and human Me275 cancer cells treated with water-soluble cholesterol/methyl-β-cyclodextrin complex (Chol) or methyl-β-cyclodextrin (MeβCD) in vitro (e, n = 3) and in vivo (f, B16F10, n = 5). Data are from one representative of at least two independent experiments with biological replicates. P values were determined by unpaired Student’s t-test. Error bars represent s.e.m. MFI, mean fluorescence intensity; NS, not significant.

1.7-fold increase in global cholesterol levels compared with adjacent skin and muscle tissues, respectively, as quantified by an Amplex Red cholesterol assay (Fig. 1b). Cholesterol levels were also upregulated in several human tumour biopsies from various cancers, including small cell lung cancer, colon cancer, squamous cell lung cancer, and liver cancer (Supplementary Fig. 1), suggesting a possibly common signature of cholesterol dysregulation in diverse cancer types. Further, single-cell analyses by flow cytometry revealed that cancer cells in a 4T1 murine breast tumour (defined by tdTomato+) displayed a 2.9-fold greater amount of plasma membrane cholesterol compared with the tumour-infiltrating leucocytes (defined by CD45.2+) (Fig. 1c). In addition, EG7 cancer cells, a murine T lymphoma cell line, had 1.7-fold more cholesterol in the plasma membrane than normal murine T cells (Fig. 1d).

Next, we sought to tune the plasma membrane cholesterol level to control cell stiffness. MeβCD, a biocompatible compound widely used as a drug solubilizer in the clinic24, has been reported to scavenge cholesterol through host–guest interaction25. We therefore used MeβCD to extract membrane cholesterol from cancer cells. Upon MeβCD treatment at a concentration of 5 mM for 30 min, the membrane cholesterol level of B16F10 cancer cells dropped markedly to only 16.0% of the native state, whereas the intracellular cholesterol level showed much less alteration (Fig. 1e). Similarly, the membrane cholesterol level of cancer cells could be lowered down to 44% of the original level in vivo through a single intratumoral (i.t.) injection of MeβCD (Fig. 1f). In addition, supplementing the plasma membrane with cholesterol using a water-soluble cholesterol/MeβCD complex increased the membrane cholesterol level by 56% (Fig. 1e). Similarly, the plasma membrane cholesterol level was manipulated in various murine cancer cell lines, including a murine lymphoma cell line expressing ovalbumin (EG7-OVA) and a murine colon cancer cell line (MC38), as well as a human melanoma cell line (Me275) (Fig. 1e and Supplementary Fig. 2). Notably, these treatments showed no direct impact on the viability or apoptosis of the cancer cells (Supplementary Fig. 3).

Control of cancer-cell stiffness by manipulating membrane cholesterol levels. To examine whether membrane cholesterol level indeed influences cancer-cell mechanical properties, we directly measured single-cell cortical stiffness using atomic force microscopy (AFM)26 (Fig. 2a). We found that cholesterol-supplemented and -depleted B16F10 cancer cells exhibited 40% lower and 2.4-fold higher cortical stiffness, respectively, compared with the untreated cells (Fig. 2b). To confirm the results, we used another well-established technique for the measurement of cell mechanics, optical tweezer27, to probe the cortical stiffness of cancer cells (Fig. 2c). In line with AFM measurements, membrane cholesterol supplementation or depletion markedly decreased or increased cortical stiffness of both murine B16F10 and human Me275 cancer cells (Fig. 2d). To investigate whether the results of single-cell measurements could be extrapolated to cell populations, we employed a recently reported high-throughput microfluidic technique called deformability cytometry to measure cellular deformation (Supplementary Fig. 4a–c), which is correlated with cellular stiffness (higher deformation correlates with lower stiffness, and vice versa)28. Murine B16F10 and human Me275 cancer cells showed markedly reduced cellular deformation after MeβCD treatment, suggesting increased cellular stiffness (Fig. 2e–g). In contrast, cholesterol supplementation in B16F10 or Me275 cancer cells increased their cellular deformation (Fig. 2e–g). A similar trend was noted
in various cancer cell types (Supplementary Fig. 4d). These results provide evidence that cholesterol enrichment in the plasma membrane contributes to cancer-cell softening, and cholesterol depletion via MeβCD treatment could increase cancer-cell stiffness.

Cancer-cell softness impairs T-cell-mediated cytotoxicity. We next investigated whether cellular softness imparts resistance to T-cell-mediated cytotoxicity. As culture substrates influence cell mechanics29, we first prepared polyacrylamide (PA) hydrogels of physiologically relevant Young’s modulus as substrates mimicking the tumour mechanical microenvironment (Supplementary Fig. 5b, PA-1 and 2)30. B16F10 cancer cells seeded on hydrogels with varying stiffness were co-cultured with activated Pmel CD8+ T cells (T-cell receptor (TCR) transgenic T cells that can recognize the gp100 antigen in B16F10 cancer cells). In accordance with a recent report18, lysis efficiency increased with higher substrate stiffness (Fig. 3a). When supplemented with exogenous cholesterol, B16F10 cancer cells seeded on a hydrogel substrate with stiffness of 143 kPa or a glass substrate (~3 GPa31) survived in higher numbers compared with the native cells, suggesting that softened cancer cells were

**Fig. 2** | Cancer-cell stiffness can be manipulated via supplementation or depletion of cholesterol in the cell membrane. **a**, Schematic illustration of the correlation between cellular stiffness and membrane cholesterol level. **b**, Relative cortical stiffness determined by nanoindentation measurements using AFM for native, Chol-, or MeβCD-treated B16F10 cancer cells (n=9–10 individual cells). Each data point is the average of at least 20 force curve measurements of a single cancer cell. Native B16F10 cancer cells serve as a standard (100%). Error bars represent s.e.m. **c**, Schematic illustration of the optical tweezer setting for cell cortical stiffness measurement. **d**, Cortical stiffness of native, Chol-, or MeβCD-treated murine B16F10 and human Me275 cancer cells measured by the optical tweezer (n=14–17 individual cells). **e–g**, Cellular deformation was measured using deformability cytometry to compare cellular stiffness in a high-throughput manner. Shown are representative scatter plots (e; sample size indicated, outliers not shown) and quantitative deformation of native, Chol-, or MeβCD-treated murine B16F10 (**e**) and human Me275 (**g**) cancer cells. In all the violin plots (**d**, **f**, **g**), the middle solid line shows the median, and the lower and upper dashed lines show the 25th and 75th percentiles, respectively. P values were determined by unpaired Student’s t-test. a.u., arbitrary units.
more resistant to T-cell-mediated cytotoxicity (Fig. 3a). To validate this observation in vivo, C57BL/6J mice bearing subcutaneous (s.c.) B16F10 tumours were administered with exogenous cholesterol through i.t. injections every other day (2 mg × 8) (Supplementary Fig. 6a). The increased cholesterol level in tumours dampened the antitumour efficacy of adoptive transfer of Pmel CD8+ T-cell-mediated cytotoxicity in vitro and in vivo. a, Lysis percentage of B16F10 cancer cells pre-treated with Chol (softened) or PBS (native) and co-cultured with activated Pmel CD8+ T cells at an E:T ratio of 10:1 for 5 h (n = 3). b, Relative membrane cholesterol levels of B16F10 cancer cells with ACAT1 KD and ACAT1 OE (n = 3). Native B16F10 cancer cells serve as a standard (100%). c, Cortical stiffness of native, ACAT1 KD, and ACAT1 OE B16F10 cells measured by the optical tweezer (n = 19-21 individual cells). In the violin plots, the middle solid line shows the median, and the lower and upper dashed lines show the 25th and 75th percentiles, respectively. d, Representative scatter plots for native, ACAT1 KD and ACAT1 OE B16F10 cells analysed by deformability cytometry, and their 50%-density contour plots (the inner contours correspond to 95% event density), with iso-elasticity lines dividing the diagrams into areas of different stiffness. e, Lysis percentage of ACAT1 KD and ACAT1 OE B16F10 cancer cells after 5 h co-culture with Pmel CD8+ T cells (n = 4). Data are from one representative of at least two independent experiments with biological replicates (a, b, e). f–h, Mice bearing native, ACAT1 KD or ACAT1 OE B16F10 tumours were treated with adoptive transfer of Pmel CD8+ T cells (5 × 10^6 per mouse), as outlined in the experimental scheme (f) (n = 5 and 10 animals for PBS- and ACT-treated groups, respectively). Shown are tumour growth curves (g) and survival curves (h) of pooled data from two independent experiments with biological replicates. P values were determined by unpaired Student’s t-test (a–c, e), two-way analysis of variance (g), or log-rank test (h). Error bars represent s.e.m.
Cancer-cell stiffening enhances the efficacy of ACT therapies. We next investigated whether such mechanical immune checkpoint can be overcome by stiffening cancer cells. We first prepared the ACAT1-overexpressing B16F10 cancer cells (denoted as ACAT1 KD B16F10) (Supplementary Fig. 7), which showed 70% lower membrane cholesterol level than native B16F10 cells (Fig. 3b). As measured by both optical tweezer and deformability cytometry, ACAT1 KD B16F10 cells with reduced membrane cholesterol level were stiffer than the native B16F10 cells (Fig. 3c,d). Indeed, ACAT1 KD B16F10 cells displayed increased susceptibility to T-cell-mediated cytotoxicity compared with the native B16F10 cancer cells (Fig. 3e). To further test this hypothesis in vivo, we inoculated mice with ACAT1 KD B16F10 tumour, which exhibited a similar growth rate as the native B16F10 tumour. However, ACT of Pmel CD8+ T cells was less effective in controlling ACAT1 KD B16F10 tumour growth compared with the native B16F10 tumour (Fig. 3g), leading to a reduced median survival of ACAT1 KD B16F10 tumour-bearing mice (Fig. 3h). Altogether, the results show that cancer cells present decreased cellular stiffness as a mechanical inhibitory pathway to evade T-cell-mediated cytotoxicity in vitro and in vivo.

Biochemical pathways of T-cell-mediated cytotoxicity are not affected by cancer-cell stiffening. By comparing IL-15SA-supported ACT therapy with the combination therapy of IL-15SA-supported ACT and MeβCD, we found that the stiffening intervention with MeβCD showed negligible effects on tumour infiltration of adoptively transferred Pmel CD8+ T cells, as well as their granzyme B production, polyfunctionality, proliferative capacity, or exhaustion phenotypes (Fig. 4f–j and Supplementary Fig. 12b). Further flow-cytometry analyses revealed that MeβCD treatment had almost no impact on the counts and phenotypes of tumour-infiltrating endogenous CD8+ T cells (Supplementary Fig. 13) or other immune cells, including regulatory T cells, B cells, natural killer cells, macrophages, dendritic cells, and myeloid-derived suppressor cells (Supplementary Fig. 14). To further examine the potential effects of MeβCD on T cells, we performed an in vitro experiment by co-culturing Pmel CD8+ T cells and B16F10 cancer cells in the presence of MeβCD. While MeβCD enhanced T-cell-mediated killing of cancer cells, it had negligible influences on T-cell proliferation or degranulation (Supplementary Fig. 15). Altogether, these results suggest that the stiffening intervention with MeβCD markedly enhances the efficacy of ACT immunotherapy against solid tumours, an effect that is not ascribed to the alteration of immune cell infiltration, proliferation or effector functions in tumours.

To elucidate the mechanism by which the stiffening intervention with MeβCD enhances tumour control by T cells, we performed mechanistic studies on different pathways that T cells exploit to kill cancer cells. These pathways include the Fas protein (also called CD95)–Fas ligand (FasL) interactions, the secretion of effector cytokines, such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), and the granule exocytosis of cytolytic proteins (for example, perforin and granzymes) (Fig. 5a)⁵⁴,⁵⁵. As TCR activation depends on cognate antigen recognition, we first validated that OVA antigen presentation on B16F10-OVA cancer cells, NFAT activation (as indicated by IL-2 production)⁵⁶, and phosphorylation levels of ZAP70, Erk1/2, and NF-κB (as indicated by p65 phosphorylation)⁵⁷ as the downstream markers of TCR signalling were not affected by the MeβCD treatment of cancer cells (Fig. 5b,c and Supplementary Fig. 16a–c). Next, we co-cultured MeβCD-stiffened B16F10 cancer cells with activated Pmel CD8+ T cells to examine whether MeβCD treatment of cancer cells altered any of the abovementioned biochemical cytotoxic pathways. Proliferation, activation, and exhaustion phenotypes were unchanged in T cells co-cultured with stiffened B16F10 with or without daily administration of MeβCD (i.t., 1 mg × 10) (Fig. 4c). ACT therapy supported by IL-15SA delayed tumour growth but failed to achieve durable control of tumour progression in all treated mice. In contrast, the combination therapy of IL-15SA-supported ACT and cancer-cell stiffening intervention using MeβCD led to the complete eradication of 5 out of 12 tumours and durable cures in 41.7% of treated mice (Fig. 4d,e). Similarly, ACT plus MeβCD treatment without IL-15SA also induced substantial tumour regression and prolonged the survival of treated mice compared with ACT alone (Supplementary Fig. 10). In addition, the stiffening intervention with MeβCD also effectively reduced the tumour burden in mice bearing s.c. EG7-OVA lymphoma tumours when combined with ACT of OT-I CD8+ T cells supported by IL-15SA (Supplementary Fig. 11), suggesting that overcoming the mechanical immune checkpoint may be a versatile therapy for different types of cancers. Notably, the stiffening intervention using MeβCD did not cause any side effects, such as body weight drop, splenomegaly, or increased infiltration and activation of CD8+ T cells in the spleens (Supplementary Fig. 12). MeβCD alone as a monotherapy showed no therapeutic efficacy, indicating that the presence of antigen-specific cytotoxic T cells was necessary for enhanced killing of stiffened target cells (Fig. 4d,e).
Cancer-cell stiffening by MeβCD enhances the efficacy of ACT immunotherapy. a, Lysis percentage of B16F10 cancer cells pre-treated with MeβCD (stiffened) or PBS (native) and co-cultured with activated Pmel CD8+ T cells at an E:T ratio of 10:1 for 5 h (n = 5). b, Lysis percentage of EG7-OVA cancer cells pre-treated with MeβCD (stiffened) or PBS (native) and co-cultured with activated OT-I CD8+ T cells at indicated E:T ratios for 5 h (n = 3).

Data in a and b are from one representative of at least three independent experiments with biological replicates. c, B16F10 tumour-bearing mice were treated with adoptive transfer of Pmel CD8+ T cells (5 × 10⁶ per mouse) adjuvanted by interleukin-15 super-agonist (IL-15SA, 10 μg per injection) with or without daily MeβCD administration (1 mg per injection) as outlined in the experimental scheme (c). Mice receiving injections of PBS or MeβCD only serve as controls (n = 12 animals per group). Shown are survival curves (d) and individual tumour growth curves (e, the number of mice with durable responses out of all treated mice is shown) of pooled data from two independent experiments with biological replicates.

f–j, Tumour-infiltrating Pmel CD8+ T cells were analysed by flow cytometry on day 14 (experimental scheme is shown in Supplementary Fig. 12b). Shown are counts (f) and frequencies of granzyme B (GrzmB)+ (g), polyfunctional (h), and PD-1+ (j), as well as Ki67 expression level (i) of tumour-infiltrating Pmel CD8+ T cells (n = 6 animals per group).

Data are from one representative of two independent experiments with biological replicates. P values were determined by unpaired Student’s t-test (a,b,f–j) or log-rank test (d). Error bars represent s.e.m.
granzyme B production revealed that MeβCD treatment of cancer cells had negligible influence on the granule exocytosis activity of the cytotoxic T cells in the co-culture (Fig. 5h,i and Supplementary Fig. 16i). On the basis of these results, we excluded the known biochemical pathways as the major underlying mechanisms of the enhanced T-cell cytotoxicity against stiffened cancer cells.
Cancer-cell stiffening augments cellular forces and cytotoxicity mediated by T cells. These findings motivated us to investigate whether biomechanical factors contribute to the enhanced T-cell-mediated killing. We first incubated MeβCD-stiffened B16F10 cancer cells with perforin, a pore-forming effector protein, in the absence of T cells, and found that cancer-cell stiffening had no impact on perforin-mediated lysis, suggesting that the enhanced cytotoxicity against stiffened cancer cells is T-cell-dependent (Fig. 5I). T-cell forces have been reported to increase membrane tension to promote pore-formation induced by perforin on target cells18. To provide direct evidence that cytotoxic T cells exert increased forces on stiffer substrates, we used traction force microscopy (TFM)38 to measure forces exerted by primary T cells on hydrogel substrates of various stiffness. We synthesized PA hydrogels with Young’s modulus ranging from 55 kPa to 143 kPa, and used primary T cells from Pmel CD8+ T cells to measure forces on these substrates. We found that T-cells exerted increased forces on stiffer substrates, as measured by traction force microscopy (TFM)38. We also measured the relative total fluorescence intensity of F-actin at the immunological synapse (IS) in activated T cells on stiffer substrates, and found that the relative total fluorescence intensity of F-actin at the IS increased with increasing substrate stiffness (Fig. 6d). We also measured the MFI of pPyk2 in activated Pmel CD8+ T cells co-cultured with native, Chol-treated (softened), or MeβCD-treated (stiffened) B16F10 cancer cells, and found that the MFI of pPyk2 increased with increasing substrate stiffness (Fig. 6f). Lysis percentage of native and stiffened B16F10 cancer cells co-cultured with activated T cells (E:T ratio of 10:1), which were pre-treated with LatA (g) or Bleb (h), was also measured. We found that lysis percentage of stiffened B16F10 cancer cells was higher than that of native B16F10 cancer cells, and that lysis percentage increased with increasing substrate stiffness. P values were determined by Kruskal–Wallis test (c, e) or unpaired Student’s t-test (f–h). Error bars represent s.e.m. in the violin plots (c, e), the middle solid line shows the median, and the lower and upper dashed lines show the 25th and 75th percentiles, respectively. All data are from one representative of at least two independent experiments with biological replicates.
Evasion from T-cell killing

Fig. 7 | Illustration of mechanical immuno-suppression induced by the softness of cancer cells. Stiffening the cancer cells enhances cancer cell killing by T-cells.

ranging from 260 to 890 Pa (Supplementary Fig. 5b, PA-3–5), a range representative of the physiological stiffness of cancer cells39–41. Upon TCR triggering by anti-CD3 and anti-CD28 antibodies coated on the hydrogel surface, T-cell forces were measured by quantifying the displacement of the embedded fluorescent beads in hydrogels (Supplementary Fig. 17a). Pmel CD8+ T cells exerted markedly higher cellular forces on stiffer substrates (Fig. 6a,b), with average values of 0.5, 1.0, and 1.8 nanonewton per cell on PA hydrogels of 260, 510, and 890 Pa, respectively (Fig. 6c). When the coated antibodies were replaced by anti-CD45 antibody (a non-stimulatory antibody) or T cells were pre-treated with latrunculin A (LatA, a potent inhibitor of actin polymerization), the cellular forces dropped substantially (Supplementary Fig. 17b). Confocal fluorescence imaging of Pmel CD8+ T cells on the same PA hydrogels revealed the presence of a filamentous actin (F-actin)-rich peripheral structure across the T-cell–hydrogel interface, which represented the formation of immunological synapse (Fig. 6d). F-actin accumulation in this synapti
cic pattern was notably increased on stiff hydrogel substrates (Fig. 6e). As F-actin polymerization is essential for cellular-force generation42, this observation, along with the TFM result, indicates that Pmel CD8+ T cells generate higher mechanical stress on stiffer surfaces. It has been reported that phosphorylation of proline-rich tyrosine kinase 2 (Pyk2), a member of focal adhesion kinase family, positively correlates with cellular forces exerted by primary T cells43. To compare the T-cell forces exerted on target cells of various stiffness, we measured the phospho-Pyk2 (pPyk2) levels in Pmel CD8+ T cells co-cultured with native, softened, or stiffened B16F10 cancer cells. A higher level of pPyk2 was induced in Pmel CD8+ T cells co-cultured with stiffened B16F10 cancer cells compared with the native or softened cancer cells (Fig. 6f), suggesting that T cells indeed exerted higher cellular forces against stiffened cancer cells. Taken together, these results show that T cells exert higher forces against a stiffer surface upon TCR triggering.

To determine whether enhanced T-cell forces played an important role in augmented cytotoxicity, we pre-treated T cells with LatA to inhibit actin polymerization and hence T-cell forces. T cells pre-treated with LatA exerted greatly reduced cellular forces even at 5 h post treatment (Supplementary Fig. 17c). As a result, the percentage of cancer-cell lysis was substantially reduced on all hydrogel substrates (Fig. 6g) compared with the results shown in Fig. 4a. Importantly, the cytotoxicity enhancement observed following cancer-cell stiffening was completely abrogated on all substrates (Fig. 6g). Similarly, pre-treatment of T cells with blebbistatin (Bleb, a myosin II inhibitor that inhibits T-cell contractility44) or Mycaloline B (MycB, an irreversible cytoskeleton inhibitor that covalently binds to globular actin (G-actin) for inhibiting actin polymerization and thus cellular forces44,45) (Supplementary Fig. 17d) led to the complete abrogation of enhanced lysis of stiffened cancer cells (Fig. 6h and Supplementary Fig. 18). Notably, these inhibitors showed no direct effects on T-cell viability or apoptosis at the concentrations used (Supplementary Fig. 19). These results reveal that the augmented killing of stiffened target cells was mediated by cellular forces exerted by T cells.

Discussion

Discovering and targeting new immune checkpoints have the potential to improve patient response rates to cancer immunotherapy. Here we identified cellular softness as an immune checkpoint of biomechanical basis that is employed by cancer cells to impair T-cell forces at the immunological synapse and therefore to evade antitumour immunity (Fig. 7). By stiffening cancer cells through depletion of cancer-cell-membrane cholesterol, we show that the mechanical immune checkpoint could be overcome to enhance T-cell forces and cytotoxicity, leading to tumour clearance and durable responses in preclinical mouse tumour models when combined with ACT therapy (Fig. 7).

Specifically modulating cell mechanics in vivo is the key to clinical applications but is still challenging. As a proof-of-concept, we used an ex vivo genetic modification approach to regulate the membrane-branched cholesterol levels specifically in cancer cells without perturbing tumour-infiltrating immune cells such as T cells. Cholesterol in T cells has been reported to be important in enhancing TCR clustering, and thus TCR signalling upon antigen stimulation15. We employed intratumoural injection of MeβCD to transiently deplete cholesterol from plasma membranes. In these experiments, we found that injected MeβCD had a negligible influence on the functions of tumour-infiltrating T cells in vivo probably because the depletion of cholesterol by MeβCD was transient and less potent compared with genetic modification of T cells in previous reports14. Future work to target reagents for mechanical modulation, such as MeβCD, specifically to cancer cells using biomaterial-assisted delivery strategies would be necessary to minimize any undesired side effects46.

We found that T-cell forces were critical for the enhanced vulnerability of stiffened cancer cells to T-cell-mediated cytotoxicity, as inhibition of T-cell forces completely abrogated such effects. In addition, TFM measurement showed that T cells exerted higher cellular forces against flat substrates with increased stiffness (from 260 to 890 Pa), which mimics the stimulatory surface of target cells. To closely recapitulate the spatial features of the target-cell surface, including the curvature, a recently developed TFM technique based on spherical microparticles may better map the dynamic forces at the T-cell immunological synapse in the future22. Degranulation and cytokine production of CD8+ T cells are less stiffness-dependent than those of CD4+ T cells, particularly on substrates of low stiffness (<8 kPa)30,47,48. Consistent with previous reports, we found that cancer-cell stiffening via membrane cholesterol depletion had a negligible effect on degranulation or cytokine production of CD8+ T cells. Recently, overexpression of myocardin-related transcription factors (MRTFs) was shown to increase cancer-cell stiffness by inducing rigidification of filamentous actin, and promote degranulation and cytokine production in cytotoxic CD8+ T cells49. These results suggest that different target-cell stiffening methods (membrane cholesterol depletion vs intracellular cytoskeleton rigidification) may result in different T-cell responses, for which the underlying mechanism is still unknown. Nevertheless, as cellular stiffness is contributed jointly by both cell cortex and cytoskeleton50, modulation of both components holds promise in overcoming the mechanical immune checkpoint for enhanced cancer immunotherapy.

Despite efforts in searching for and investigating immune checkpoints on the basis of biochemical signals, much less is known about how biomechanical cues and interactions could potentially regulate
immune responses against diseases such as cancer. The cancer–immunity interactions are multidimensional, involving not only biochemical but also substantial biophysical signals. Our studies provide insight into the multidimensional mechanisms of immune suppression in tumours. The growing knowledge in fundamental mechano-immunology provides the basis for developing new engineering approaches to modulate biomechanical cues for enhanced antitumour immunity. Leveraging cancer-cell mechanics and T-cell forces, as demonstrated in this study, may provide new therapeutic strategies in addition to conventional biochemical modulation. Therapeutically targeting both biochemical and mechanical immune checkpoints could potentially benefit patients broadly with cancer immunotherapies.

Methods

Animals, cell lines, and reagents. All the mouse studies were approved by the Swiss authorities (Canton of Vaud, animal protocol ID 3206 and 3535) and performed in accordance with guidelines from the Center of Phenogenomics (CPG) in EPFL. Six- to eight-week-old female Thy1.2 C57BL/6 (C57BL/6J) mice and BALB/cByJ (BALB/c) mice were purchased from Charles River Laboratories. T-cell receptor (TCR)-transgenic Thy1.1 pmel-1 (Pmel) mice (B6.Cg-Ty1/Ty1(Cg)Tg(TcraTcrob)6Mjt/J) and TCR transgenic OT-I mice (C57BL/6-Tg(TcraTcrob)10Mjt/J) were purchased from Jackson laboratory and maintained in the animal facility in the CPG in EPFL. B16F10 murine melanoma cells and EL4 murine lymphoma cells expressing ovalbumin (EG7-OVA) were originally acquired from the American Type Culture Collection (ATCC). MC38 murine colon cancer cells expressing human epidermal growth factor receptor 2 (MC38-HER2) and Me275 human melanoma cells expressing HER2 (Me275-HER2) were kindly provided by the Pedro Romero Lab (UNIL, Switzerland). B16F10 murine melanoma cells expressing ovalbumin (B16F10-OVA) and 4T1 murine breast cancer cells expressing luciferase and tdTomato fluorescent protein (4T1-Fluc-tdTomato) were kindly provided by the Darrell Irvine Lab (EPFL, Switzerland). B16F10, B16F16-OVA, MC38-HER2, and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Thermo Fisher) supplemented with foetal bovine serum (FBS) (10% v/v%), HEPES (1 v/v%, Gibco), penicillin/streptomycin (1 v/v%), and L-glutamine (2 mM). MC38, B16F10-OVA, and 4T1 murine breast cancer cells expressing luciferase and tdTomato fluorescent protein were cultured in RPMI 1640 medium (Gibco) supplemented with FBS (10% v/v%), L-glutamine (2 mM), penicillin/streptomycin (1 v/v%), and L-glutamine (2 mM). For culturing EG7-OVA cells, 4G18 (Geneticin) (0.4 mg·mL⁻¹) was supplemented to maintain OVA expression. 4T1-Fluc-tdTomato cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco) supplemented with FBS (10% v/v%) and penicillin/streptomycin (1 v/v%).

Filipin III (from Streptomyces filipinensis), methyl-β-cyclodextrin (MεCD), water-soluble cholesterol/MεCD complex (Chol), blebbistatin (Bleb), glutaraldehyde solution (25 wt% in H₂O), Triton X-100, 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), propidium iodide solution (1 mg·ml⁻¹ in PBS) in PBS) at 4 °C, Hoechst 33342, acrylamide, N-sulfosuccinimidyl-6-azido-2-nitrophenylamino) hexanoate (Sulfo-SANPAH), deoxyribonuclease I (DNase I, from bovine pancreas), dispase II, hyaluronidase, cholesterol oxidase (4 μg·μl⁻¹ in Dulbecco’s modified Eagle’s medium (DMEM)) (Gibco, Thermo Fisher) supplemented with foetal bovine serum (FBS) (10% v/v%), Gibco and penicillin/streptomycin (1 v/v%), and β-mercaptoethanol (0.1% v/v%, Gibco). For culturing EG7-OVA cells, 4G18 (Geneticin) (0.4 mg·mL⁻¹) was supplemented to maintain OVA expression. 4T1-Fluc-tdTomato cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco) supplemented with FBS (10% v/v%) and penicillin/streptomycin (1 v/v%).

For flow cytometry analyses, fluorocently labelled anti-phospho (Tyr319/Tyr352)-ZAP70/Syk antibody (n3kobu5) and goat anti-rabbit IgG (H + L) secondary antibody (polyclonal) were purchased from Invitrogen. Fluorocently labelled Annexin V, and antibodies including anti-mCD4 (RM4–5), anti-mCD8 (YTS156.7.7), anti-mCD3e (17A2), anti-IFN-γ (XMGL2.1), anti-TNF-α (MP6–X22), anti-IL-2 (JE6–SH4), anti-granzyme B (GB11), anti-CD107a (1D4B), anti-CD69 (HI.12F3), anti-PD-1 (29F1A12), and anti-K6 (16A7, 16A8, against Foxp3 (MF-14), anti-NK1.1 (PK36), anti-I-A/I-E (M5/114.15.2), anti-F4/80 (BM8), anti-CD19 (B6.8C5.6), anti-Gr-1 (RB6–8C5), anti-CD3e (17A2), and anti-CD11c (N418), anti-Siglec-F (S1702L), anti-Thy1.1 (OX-7), and anti-CD45.2 (104), anti-phospho (Thr202/Tyr204) Erk1/2 (4B1869), anti-Fas (MFL3), anti-H-2Kb-SIINFEKL (25-D1.6), anti-Fas (SA3678), and anti-PD-1 (10F9G2) were purchased from Biolegend.

Histological analyses. Mouse samples including tumour tissues and adjacent tissues (skin and muscle) were collected from C57BL/6J mice 10 days after subcutaneous inoculation with B16F10 cancer cells (5 x 10⁴). The collected tissues were embedded in O.C.T. compound (Tissue-Tek, Sakura Finetek) and frozen with liquid nitrogen for cryosection with Leica CM3050 cryostat (Leica Microsystems). Cryosections collected on slides were thawed and hydrated in phosphate-buffered saline (PBS, Gibco) for 15 min at room temperature. The section slides were then stained with Filipin III (100 µg·ml⁻¹ in PBS) for 1 h followed by rinsing with PBS twice. The corresponding adjacent section slides were sent to the Histology Core Facility at EPFL for hematoxylin and eosin (H&E) staining. The slide images were acquired using a confocal microscope (LSM700, Zeiss) and processed using ImageJ. All human biopsy samples were obtained from West China Hospital (Chengdu, China) following the approved protocol (No,120, 2016). Informed consent was obtained from the West China Hospital ethics committee. Briefly, the collected cancer samples from human patients were embedded in O.C.T. compound (Tissue-Tek) and frozen with liquid nitrogen for cryosection with Leica CM3050 cryostat. Cryosections were performed in O.C.T. compound (Tissue-Tek) processed using ImageJ. The slide images were acquired using an inverted microscope (Eclipse Ti-U, Nikon) and processed using ImageJ.

Characterization of global cholesterol levels in tumour, skin, and muscle tissues. B16F10 tumour, skin or muscle tissues adjacent to the tumour were collected separately from tumour-inoculated mice and digested with tissue digestion buffer (0.1 mg·ml⁻¹ DNase I, dispase II, and hyaluronidase, and 1 mg·ml⁻¹ collagenase IV in RPMI 1640 medium) on a shaker at 37 °C for 1 h. The tissue fluid passing through a cell strainer (70 µm, Thermo Fisher) was collected. The cholesterol (1.2, v/v) for cholesterol extraction and shaken at room temperature for 2 h. Afterwards, the organic phase containing cholesterol was collected and the solvent was evaporated under vacuum. Finally, the cholesterol in each sample was quantified using the Amplex Red cholesterol assay kit (Invitrogen) according to the manufacturer’s recommended protocol.

Filipin staining of cholesterol for flow cytometry analyses. Tissue samples were collected from BALB/c mice 12 after subcutaneous inoculation with 4T1-Fluc-tdTomato cancer cells (5 x 10⁴), and digested with tissue digestion buffer on a shaker at 37 °C for 45 min. After passing through a cell strainer (70 µm), the red blood cells were lysed with ACK lysis buffer (Gibco) at room temperature for 5 min. The collected cells were then stained with Filipin (10 µg·ml⁻¹ in PBS) at 4 °C for 30 min. After washing with PBS (0.2 v/v% BSA), the cells were resuspended in propidium iodide solution (10 µg·ml⁻¹ in PBS) for flow cytometry analyses. Similar Filipin staining was performed with EG7-OVA cancer cells and activated CD8⁺ T cells to compare their membrane cholesterol levels.

Modulation of cholesterol levels in plasma membrane of cancer cells. To deplete cholesterol from plasma membrane, B16F10 (or EG7-OVA, MC38-HER2, and Me275-HER2) cancer cells were incubated with DMEM supplemented with MεCD (5 mM) at 37 °C for 30 min, and then washed with PBS twice. To supplement cholesterol, B16F10 (or EG7-OVA, MC38-HER2, and Me275-HER2) cancer cells were treated with Chol (5 mM) in DMEM at 37 °C for 30 min, and then washed with PBS twice.

Generation of ACAT1 knock-down and overexpressing B16F10 cancer cell lines. Lentiviral plasmids containing the ACAT1 knock-down and overexpression constructs were generated by standard molecular cloning methods. To generate ACAT1 knock-down cells, the short-hairpin RNA target sequence (5'-CCACCGAGACGATCAAATCATAT-3') was cloned into the pLKO vector with the AgeI/EcoRI sites. To generate ACAT1-overexpressing cells, cobon-optimized complementary DNA encoding ACAT1 (NM_009203.3) was synthesized by Twist Biosciences and cloned into the lentiviral expression vector S002 by Gibson assembly cloning.

Lentinivus was produced by transient transfection of HEK293T cells with the S002 or pLKO1 transgene expression vectors, the pDela8.9 packaging plasmid and the VSV-G envelope plasmid. In brief, HEK293T cells were transfected with a mixture of plasmid DNA (VSV-GpDela8.9.S002) or (pLKO1) with a weight ratio.
of 1:2:3) assembled in calcium-phosphate particles. After overnight incubation, the medium was replaced with normal culture medium. Supernatants containing viral particles were collected 48 and 72 h after transfection and filtered through a 0.45-µm filter. Untreated 293T supernatant supplemented with polyethyleneimine (10 µg µl⁻¹) was added to B16F10 cells, and contact of viral particles with cells was ensured by centrifugation (2,000 r.p.m., 1 h). One day after transduction, stably transduced B16F10 cells were subjected to puromycin selection (0.5 µg µl⁻¹) for 2 weeks.

Quantification of intracellular and plasma membrane cholesterol levels. The total cellular cholesterol level was first quantified using the Amplex Red cholesterol assay kit. In brief, B16F10 (or EG7-OVA, MC38-HER2, and Me275-HER2) cancer cells were fixed with glutaraldehyde (1.0 wt% in PBS) and the total cholesterol was extracted with methanol/chloroform (1:2, v/v) under sonication. After removal of the organic solvent under vacuum, the cholesterol level was measured immediately post Me275-HER2 βCD treatment.

Viability and apoptosis assays of cancer cells. The viability and apoptosis of MeJCd-treated cancer cells were evaluated using Annexin V and 7-AAD (0.5 µg µl⁻¹) solution (5 mM) at 37 °C, followed by the addition of 55 v/v% Percoll at the bottom. Annexin V and 7-AAD stained with fluorescently labelled Annexin V and 7-AAD (0.5 µg µl⁻¹) in PBS were then added to the cells. For quantification, the dead (Annexin V and 7-AAD −), early apoptotic (Annexin V and 7-AAD +), and late apoptotic/necrotic (Annexin V and 7-AAD −) cancer cells were quantified using flow cytometry analysis.

Measurement of cell cortical stiffness by AFM. The measurement of cell cortical stiffness by AFM was performed using the Nanoscope Analysis (Bruker) software. In brief, cancer cells were mobilized using an XY stage until the cell of interest was placed under the AFM objective (20×) for live-cell imaging. The samples were stained with fluorescently labelled Annexin V and DAPI for cell identification and visualized using an inverted microscope (Nikon) equipped with a high-speed camera (VEO640L, Phantom). To ensure that cell deformation was measured accurately, the flow rate was set to 4 µl min⁻¹ (B16F10, EG7-OVA, and MC38-HER2). The stiffness force-displacement curve was recorded using a customized Dimension Icon AFM (Bruker) with a 10-W, 1,064-nm laser beam (10 µm, 5.14 N m⁻¹), and dried in air for 30 min. To prepare PA hydrogels of different stiffness (PA-1 µm, 300 µm, and 300 µm, respectively), the samples were compressed using a 1 kg weight overnight at 100 °C to ensure firm and robust attachment.

Deformability cytometry (DC). The DC setup was built following a published report. A 4 inch silicon wafer was selectively etched using photolithography and deep reactive ion etching for the fabrication of the microfluidic device. The height, width, and length of the constriction area were measured using a mechanical profiler (Dektak XT, Bruker) at 30 µm, 300 µm, and 300 µm, respectively. The chosen geometry ensured that the cells were deformed properly. The projected areas of cells were 90% to 50% of the cross-sectional area of the constriction zone. Devices were cast using a 1:1 mixture of polydimethylsiloxane (Sylgard 184, Dow Corning) and 5% acrylamide solution. The microfluidic device was sealed by curing the prepolymer overnight at 65 °C. Glass coverslips (No.1, VWR) were cleaned thoroughly by soaking them in acetone, isopropyl alcohol, and ethanol, and distilled water, and subsequently dried at 65 °C to avoid any possible leakage in the microfluidic device due to the high viscosity of the solutions and high flow rate. The surfaces of the coverslips and polydimethylsiloxane devices were treated with a plasma cleaner (PDC-002-2D; Plasma Harrick) at 29 W for 45 s before bonding. They were subsequently compressed using a 1 kg weight overnight at 100 °C to ensure firm and robust attachment.

In a typical DC measurement, cells were allowed to flow through a microfluidic channel at a flow rate of 4 µl min⁻¹ (Me275-HER2 βCD, 5.14 N m⁻¹ (B16F10, EG7-OVA, and MC38-HER2). The cells were centred in the microfluidic channel using shear flow (Supplementary Fig. 4a). The shear flow rate was set at 3 x the cell flow rates, that is, 12 µl min⁻¹ or 16 µl min⁻¹, respectively. The flow rates were controlled with a programmable syringe pump (neMESYS 290, Cetoni). Before the measurement, the cells were treated with MeJCd or Chol as mentioned above or kept in PBS without treatment. The cells were then suspended in methyl cellulose solution (0.6 v/v% in HBSS) at a concentration of 2 x 10⁶ cells per ml. The cells were then compressed through the narrow constriction zone of 30 µm x 300 µm (height x width x length), and visualized using an inverted microscope (Nikon) equipped with a 20× objective and a high-speed camera (VEO640L, Phantom). To ensure that cell deformation was measured accurately, the time-lapse movies containing several thousands of cells were analysed using a custom ImageJ macro (Bioimageing and Optics Platform, EPFL). Briefly, the cell contour was first identified on the basis of grey scale value. Subsequently, a convex hull (ImageJ) was fitted on the cells to avoid a large increase in cell perimeter. The measured projected cellular area and perimeter were used to calculate the deformation (D) defined as:

\[ D = 1 - \frac{A_{\text{contour}}}{A_{\text{hull}}} \]

where A is the projected cell surface area and l is the cell perimeter. The deformation was measured using MATLAB. In particular, cells with irregular shapes or those with poor contour identification were eliminated as proposed in the published literature. The ratio of the projected area was calculated using the hull approximation A_{hull} and the grey scales value A_{contour} as:

\[ R = \frac{A_{\text{contour}}}{A_{\text{hull}}} \]

Cells with R ≥ 1.07 were eliminated in the final analysis.

Deformations of cell populations with different cell areas were compared using iso-elasticity lines plotted using Shape-Out (Paul Müller and others, version 2.7.4). To input the data in Shape-Out, the above post-processed data in .xlsx format were converted into the H5 format using MATLAB.

Fabrication and rheological test of PA hydrogel substrates. PA hydrogel substrates were prepared using a protocol adapted from ref. 39. Briefly, a 96-well glass-bottom plate (Falcon, Corning) was treated with NaOH solution (0.1 M in H₂O, 50 µl per well) for 5 min at room temperature (Supplementary Fig. 5a). Upon removal of the NaOH solution, APTMS (20 µl per well) was applied for 3 min at room temperature. The well plate was then thoroughly rinsed with distilled water (DI water) for 5 min. The remaining APTMS was rinsed by a solution of glutaraldehyde solution (0.5 wt% in H₂O, 50 µl per well) and incubation at room temperature for 20 min. The well plate was then subsequently rinsed with DI water and dried in air for 30 min. To prepare PA hydrogels of different stiffness (PA-1 and 2 as shown in Supplementary Fig. 5b), we made the PA hydrogel precursor solutions (20 µl per well) with various remaining APMA (acryl-methacrylamide monomer and the bis-acrylamide cross-linker) concentrations. Ammonium persulphate (0.1 v/v% in final concentration) and TEMED (0.1 v/v% in final concentration) were then added to initiate the polymerization, followed by a brief vortexing and incubation at room temperature for 1 h. The hydrogel substrates were then washed with PBS (200 µl).
Activation of Pmel and OT-I CD8+ T cells. Splenectomies were performed before the experiments. To activate Pmel CD8+ T cells, a mixture of splenocytes and IL-2 was injected i.v. onto the hydrogel surface, followed by UV irradiation and washing. For OT-I CD8+ T cells, a similar procedure was followed but without MeHepes buffer, followed by washing with PBS twice. Next, a suspension of activated Pmel CD8+ T cells was fixed and permeabilized with Cyto-Fast Fix/Perm Buffer Set (Biolegend), followed by fixation and permeabilization with Fsg3/Transcription Factor Buffer Set (Biolegend), and stained with the fluorescently labelled antibodies against cytotoxicity and IFN-γ. The data was analyzed using flow cytometry.

Characterization of tumour-infiltrating immune cells by flow cytometry analyses. B16F10 melanoma cells (1 × 106) were injected subcutaneously in the right flanks of Thy1.2+ C57BL/6 mice on day 0. On day 4, activated Pmel Thy1.1+ CD8+ T cells (5 × 104) per dose in 50 μl PBS) were intratumourally injected into each of the mice. On day 9, B16F10 tumour-bearing mice received three therapeutic infusions of activated Pmel Thy1.1+ CD8+ T cells (5 × 104 per dose) on days 4, 11, and 17. The data was analyzed using flow cytometry.

Flow-cytometry analysis of cancer cells from in vitro co-culture assays. For analysis of B16F10 (or B16F10-OVA) cancer cells, only cells were treated with MeHepes buffer (50 mM) in PBS (100 μl) before incubation with 250 μl of the reagent, followed by washing with PBS twice. Next, a suspension of activated OT-I CD8+ T cells (5 × 104 per dose) in 50 μl PBS) was added to each well at an E:T ratio of 10:1. Mice were monitored as described above.

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stained with fluorescently labelled anti-PD-1, anti-CD69, anti-FasL, anti-CD107α, anti-granzyme B, anti-TNF-α, anti-IFN-γ, and anti-IL-2 antibodies for flow cytometry analysis.

To determine the protein phosphorylation in T cells, the plate for co-culture was centrifuged at 1,500 r.p.m. for 2 min, followed by a brief co-incubation for 5 min. To detect phosphorylation of TCR signalling proteins ZAP70, Erk1/2, and NF-κB, T cells were fixed with a parafilmdehyde solution (1.5 wt% v/v in PBS), and then permeabilized by resuspending in ice-cold methanol at 4°C for 10 min, followed by washing with PBS (0.2 wt% v/v BSA). Next, T cells were stained with fluorescently labelled anti-phospho-ZAP70, anti-phospho-Erk1/2, or anti-phospho-NF-κB antibodies for flow cytometry analysis. The mean fluorescence intensities of phospho-ZAP70, phospho-Erk1/2, and anti-phospho-NF-κB were normalized by the corresponding levels in unstimulated Pmel CD8+ T cells. To detect phosphorylation of Pyk2, T cells were fixed with parafilmdehyde solution (1.5 wt% v/v in PBS) and then permeabilized by resuspending in Triton X-100 solution (0.1 wt% v/v BSA) at 25°C for 5 min. After washing with PBS (0.2 wt% v/v BSA), T cells were stained with anti-phospho-Pyk2 antibodies and then fluorescently labelled secondary antibody for flow cytometry analysis.

In another assay, B16F10 cancer cells were seeded on a 48-well plate and incubated overnight for cell attachment. A suspension of activated Pmel CD8+ T cells in complete RPMI medium supplemented with FBS (10 wt%) contained recombinant mouse IL-2 (10 ng ml–1) and MeJCC (0.05 mM) was then added to each well at an E:T ratio of 1:1. After 48 h co-incubation at 37°C, T cells and B16F10 cells were collected for flow cytometry analysis.

Preparation of fluorescent bead-embedded PA hydrogel substrates for TFM.

To prepare biotinylated anti-CD3 and anti-CD28 antibodies for hydrogel surface coating, an NHS-biotin (10 mM) solution in dimethylsulfoxide was added to a solution of anti-CD3 or anti-CD28 antibodies (2 mg ml–1 in PBS) at 37°C. After 1 h, activated Pmel T cells were treated with LatA (2 µM in PBS) at 37°C for 10 min before being washed with PBS twice. T cells were next permeabilized by resuspending in Triton X-100 in PBS (0.1 wt% v/v) at room temperature for 5 min. After washing with PBS twice, T cells were stained with phalloidin-iFluor 488 (1/1,000 dilution according to the manufacturer’s recommended protocol) and Hoechst 33342 (5 µg ml–1 in PBS (1 wt% BSA) at room temperature in the dark for 30 min. After washing with PBS three times, Fluoromount-G mounting medium (300 µl, Invitrogen) was added on the hydrogel surface, and a coverslip (thickness 0.17 mm, diameter 25 mm) was placed on top to seal the glass bottom. The confocal images were acquired using an inverted microscope (IX83, Olympus) equipped with a spinning disk confocal scanner (CSU-W1, Visitron) and a 100/1.40 UPLSAPO objective (Olympus). T cells in Fig. 5d were imaged at high resolution (2x-stack step size, 150 nm) and deconvoluted using Huygens Remote Manager (Scientific Volume Imaging). The F-actin fluorescence images in the XZ plane (side view) were obtained by summing 20 slices in the XZ plane in the middle of the cells (Z project plugin). The intensity display settings are identical for each side-view image (1,500–25,000). The F-actin fluorescence images at the immunological synapse (IS, defined as the structure between the surface of the hydrogel and a height of ~2 µm above the surface of the hydrogel) of T cells were obtained by overlaying Z-stack slices located within the IS. The intensity display settings are identical for each IS image (0–18,000).

The total F-actin fluorescence intensity at the IS of T cells was measured using semi-automated ImageJ (National Institutes of Health, USA). Briefly, Z-stack slices located within the IS were summed (Z project plugin). The resulting image was thresholded (‘threshold’ plugin) and holes filled (‘fill holes’ plugin). Cell contours were automatically identified (‘analyse particles’ plugin). Further post-processing was performed using MATLAB. The corrected F-actin fluorescence intensity per pixel at the IS was obtained by subtracting the average background value from the average F-actin fluorescence intensity per pixel at the IS. Finally, to calculate the total F-actin fluorescence intensity at the IS, the corrected F-actin fluorescence intensity per pixel was multiplied by the cell area.

Statistical analyses. Data are presented as mean ± s.e.m. unless otherwise noted. Violin plots show frequency distribution curves created by the kernel density method in which the middle solid line shows the median, and the lower and upper dashed lines show the 25th and 75th percentiles, respectively. Statistical analysis for each experiment is specified in the corresponding figure legend. Statistical analyses were performed using GraphPad Prism 8 software. In all cases, two-tailed t-test with P<0.05 were considered significant.

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

Data availability

The data supporting the results in this study are available within the paper and its Supplementary Information. Source data for the figures are provided with this paper.

Code availability

Source code for the custom ImageJ macro for F-actin analysis, cellular-force calculation and deformability cytometry analysis, and custom MATLAB scripts for optical-tweezer data collection and data post-processing are available from the corresponding author on reasonable request. Shape-OUT (version 2021)
2.4) for deformability cytometry analysis is available at https://github.com/ZELLMECHANIK-DRESDEN/ShapeOut2.

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Author contributions
K.L. and L.T. conceived the study and designed the experiments. K.L., A.K., M.K., L.B., Y.H., V.C., M.G., Y.-Q.X., Y.G., M.T.M.H., Y.W., G.Z., M.G., G.E.F., and M.S.S. performed the experiments. K.L., A.K., L.T., M.S.S., M.G., and G.E.F. analysed the data. L.T. supervised the project. K.L., A.K., and L.T. wrote the manuscript. All authors edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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|     | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
|     | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
|     | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted |
|     | Give P values as exact values whenever suitable. |
|     | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
|     | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
|     | Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Flow-cytometry data collection was performed with Attune NxT Software version 3 (Invitrogen, Thermal Fischer Scientific, Waltham, MA, USA). Traction-force-microscopy data collection was performed using VisiView software (VisiView, Puchheim, Germany). Deformability-cytometry data collection was performed using Phantom Camera Control software (Phantom, Wayne, NJ, USA). Confocal imaging was performed using ZEN 2009 software (Zeiss, Oberkochen, Germany). Atomic-force-microscopy data collection was performed using NanoScope 8.15 software (Bruker, Billerica, MA, USA). Optical-tweezer data collection was performed using a customized code in MATLAB (Mathworks, Natick, MA, USA). Microplate-reader measurements were performed using Skanlt software (Thermal Fischer Scientific, Waltham, MA, USA).

Data analysis

Flow-cytometry data were analysed using FlowJo 10.7 (Tree Star, BD Biosciences, Franklin Lakes, NJ, USA). Statistical analyses were performed using GraphPad Prism 8 (GraphPad software, La Jolla, CA, USA). Image analyses were performed using ImageJ (Fiji, open-source). Image deconvolution was performed using Huygens Remote Manager (Scientific Volume Imaging, Hilversum, Netherland). Deformability-cytometry analysis was performed using Shape-Out (version 2.7.4, available at https://github.com/zellmechanik-dresden/ShapeOut2). For data post-processing, MATLAB (Mathworks, Natick, MA, USA) was used.

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

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data supporting the results in this study are available within the paper and its Supplementary Information. Source data for the figures are provided with this paper.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

| Sample size | Group sizes for the in vivo validation experiments were selected empirically on the basis of previous results of the intra-group variation of tumour growth in similar treatments. Usually 3–12 mice per group are sufficient to detect significantly biological differences with good reproducibility. For the in vitro experiments, group sizes were selected on the basis of previous publications and of prior knowledge of variation. |
| Data exclusions | Rout outlier tests were run with default parameters (Q = 1%) in Prism on all experimental data, due to inherent variability within the model system. |
| Replication | All the reported results were repeatable. Replicates were used in all experiments, as noted in the figure legends and in Methods. |
| Randomization | Age-and-sex-matched animals were used for each experiment. Mice were randomized prior to treatment. For the experiments without mice, there was no randomization of samples because these in vitro experiments were observational and replicated at least in 2 independent experiments. |
| Blinding | For the in vivo experiments, no blinding was performed owing to requirements for cage labeling and to staffing needs. The investigator needed to know the treatment for each group, especially when the experiments were performed by a single person. For the in vitro experiments, all the treatments were performed in a parallel manner without risk of bias in interpretation. All data were acquired and analysed by software with an objective standard. |

Reporting for specific materials, systems and methods

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

| Materials & experimental systems | Methods |
| n/a | n/a |
| Involved in the study | Involved in the study |
| - Antibodies | - ChIP-seq |
| - Eukaryotic cell lines | - Flow cytometry |
| - Palaeontology | - MRI-based neuroimaging |
| - Animals and other organisms | |
| - Human research participants | |
| - Clinical data | |

Antibodies

The following antibodies were purchased from BioLegend (San Diego, CA, USA): anti-CD4 (RM4-5), anti-CD8 (YTS156.7.7), anti-CD3e (17A2), anti-IFN-y (XMG1.2), anti-TNF-alpha (MP6-XT22), anti-IL-2 (JES6-5H4), anti-granzyme B (GB11), anti-IL-2 (JES6-5H4), anti-CD69 (H1.2F3), anti-PD-1 (29F.1A12), anti-ICAM1 (517B), anti-Foxp3 (MF14), anti-I-A/I-E (M5/114.15.2), anti-F4/80 (BM8), anti-CD19 (6D5), anti-CD11b (M1/70), anti-CD11c (N418), anti-Siglec-F (S17007L), anti-Thy-1.1 (OX-1), anti-FasL (MFL3), anti-H-2Kb-SIINFEKL (25-D1.16), anti-Fas (28-1)
(SA367H8), and anti-PD-L1 (10F.9G2). Anti-phospho (Tyr319/Tyr352)-ZAP70/Syk antibody (n3kobu5), anti-phospho-NF-κB p65 (Ser536) antibody (T.849.2), and goat anti-rabbit IgG (H+L) secondary antibody (polyclonal) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Anti-phospho (Tyr402)-Pyk2 antibody (R402) was purchased from Enogene (New York, NY, USA). Anti-ACAT1 polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-mouse CD3 antibody (clone 17A2), and anti-mouse CD28 antibody (clone 37.51) were purchased from Bioxcell (West Lebanon, NH, USA).

Validation

All the primary antibodies are well-recognized clones, as noted in many publications, and are commercially available and validated by the manufacturers, as indicated on their websites. These antibodies are further validated and routinely used in our lab, with good reproducibility.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
The B16F10 murine melanoma and EG7-OVA murine lymphoma cell lines were originally acquired from the American Type Culture Collection (Manassas, VA, USA). 4T1-Fluc-tomato murine breast cancer cell lines were provided by Prof. Darrell J. Irvine (MIT, USA), and generated as reported previously. The HER2-transduced MC38 murine colon cancer (MC38-HER2), the ME275 human melanoma (ME275-HER2) and the B16F10-OVA murine melanoma cell lines were obtained or generated as cited.

Authentication
None of the cell lines were authenticated in these studies. In all related studies, cell lines with a low passage number were used.

Mycoplasma contamination
All cell lines were confirmed to be free of mycoplasma.

Commonly misidentified lines (See ICLAC register)
No commonly misidentified cell lines were used.

Animals and other organisms

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

Laboratory animals
Six-to-eight-week-old female Thy1.2+ C57BL/6 (C57BL/6J) mice and BALB/cByJ (BALB/c) mice were purchased from Charles River Laboratories (Lyon, France). T-cell receptor (TCR)-transgenic Thy1.1+ pmel-1 (PMEL) mice (B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J) and TCR-transgenic OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the animal facility of the Center of PhenoGenomics in École Polytechnique Fédérale de Lausanne (EPFL). For all the adoptive-transfer experiments, donor mice and recipient mice were age-and-sex-matched. All mice were housed in the animal facility of the Center of PhenoGenomics at EPFL, and were kept in individually ventilated cages, between 19–23 °C with 45–65% humidity and with a 12 hour dark/light cycle.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from the field.

Ethics oversight
The experimental procedures in the mouse studies were approved by the Swiss authorities (Canton of Vaud, animal protocol ID 3206 and 3533) and performed in accordance with the guidelines from the Center of PhenoGenomics at EPFL.

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
Human cancer tissues were obtained from patients undergoing diagnostic biopsy at the West China Hospital (Chengdu, China).

Recruitment
All human biopsy samples were obtained without specific recruitment. It was not necessary to recruit a specific subpopulation for this study.

Ethics oversight
Written informed consent was obtained from all patients. The study was approved by the ethics committee of West China Hospital (Chengdu, China) following an approved protocol (No.120, 2016).

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

Tumours were dissected from the surrounding tissues, weighed, mechanically minced, and stirred at 1,000 rpm in RPMI-1640 medium with Collagenase Type IV (1 mg/mL, Gibco, Thermo Fisher Scientific), Dispase II (100 μg/mL, Sigma-Aldrich, St. Louis, Missouri, USA), Hyaluronidase (100 μg/mL, Sigma-Aldrich), and DNase I (100 μg/mL, Sigma-Aldrich) at 37 °C for 60 min for digestion. Red-blood-cell lysis was performed on the tumour-digestion samples with ACK Lysing Buffer (Gibco, Thermo Fisher Scientific), and then tumour-infiltrating leukocytes were enriched by density gradient centrifugation against Percoll (GE healthcare, Chicago, IL, USA) and resuspended in PBS with bovine serum albumin (0.2 wt%, Sigma-Aldrich).

Instrument

Data were collected using Attune Nxt Flow Cytometer (Invitrogen, Thermal Fischer Scientific).

Software

Flow-cytometry data collection was performed using Attune Nxt Software version 3 (Invitrogen, Thermal Fischer Scientific). Flow-cytometry data were analysed using FlowJo 10.7 (Tree Star, BD Biosciences, Franklin Lakes, NJ, USA).

Cell population abundance

Purity was determined by flow cytometry for activated primary CD8+ T-cells (> 95 %). For a typical analysis, >50k leukocytes were collected for further gating.

Gating strategy

We used standard gating strategies: gating on the typical lymphocyte population based on FSC-SSC signals, doublet exclusion based on FSC-H and FSC-A comparison, Live/Dead discrimination based on DAPI or fixable Aqua dye signals. Cell populations were identified based on the expression markers listed below. CD8 T-cells: CD45+/CD3+/CD8+; CD4 T-cells: CD45+/CD3+/CD4+; regulatory T cells: CD45+/CD3+/CD4+/FoxP3+; B cells: CD45+/CD3-/CD19+; natural killer cells: CD45+/CD3-/NK1.1+; dendritic cells: CD45+/Gr-1-/CD11b+/CD11c+/MHCII+; Tumor-associated macrophages: CD45+/Gr-1-/CD11b+/F4/80+/MHCII+; myeloid-derived suppressor cells: CD45+/Gr-1+/CD11b+; Eosinophils: CD45+/CD11b+/Siglec-F+. The gating strategy is described in Supplementary Figure 14a.

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