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## PD-1 suppresses the maintenance of cell couples between cytotoxic T cells and target tumor cells within the tumor

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### Abstract

The killing of tumor cells by CD8<sup>+</sup> T cells is suppressed by the tumor microenvironment, and increased expression of inhibitory receptors, including programmed cell death protein-1 (PD-1), is associated with tumor-mediated suppression of T cells. To discover cellular defects triggered by tumor exposure and associated PD-1 signaling, we established an ex vivo imaging approach to investigate the response of antigen-specific, activated effector CD8<sup>+</sup> tumor infiltrating lymphocytes (TILs) after interaction with target tumor cells. Although TIL–tumor cell couples readily formed, couple stability deteriorated within minutes. This was associated with impaired F-actin clearing from the center of the cellular interface, reduced Ca<sup>2+</sup> signaling, increased TIL locomotion, and impaired tumor cell killing. The interaction of CD8<sup>+</sup> T lymphocytes with tumor cell spheroids in vitro induced a similar phenotype, supporting a critical role of direct T cell–tumor cell contact. Diminished engagement of PD-1 within the tumor, but not acute ex vivo

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blockade, partially restored cell couple maintenance and killing. PD-1 thus contributes to the suppression of TIL function by inducing a state of impaired subcellular organization.

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## Introduction

Cancer cells are commonly recognized by the immune system, and immune cells constitute a large part of the tumor mass. Cytotoxic T lymphocytes (CTLs) are capable of directly killing cancer cells; however, their killing ability is widely suppressed after they have entered the tumor, where they are known as tumor-infiltrating lymphocytes (TILs). A number of key contributors to tumor-mediated immune suppression in vivo have been characterized. Most prominently, tumor-reactive T cells increase their cell surface expression of inhibitory receptors, including cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1), lymphocyte activation gene 3 (LAG3), T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), and T cell molecule with immunoglobulin and mucin domain 3 (TIM3) (1, 2). Monoclonal antibody-mediated blockade of CTLA-4 and PD-1 has yielded substantial clinical success in enhancing the antitumor immune response (3). Additional mechanisms of tumor-mediated immune suppression include the recruitment of tolerogenic immune cells, notably CD4<sup>+</sup> regulatory T cells (Tregs) into the tumor (4, 5), and the expression of suppressive soluble mediators, such as adenosine and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (6-8). Whereas it is clear that tumor cell killing is diminished due to the immunosuppressive tumor microenvironment, it is still uncertain as to which cellular steps in target tumor cell killing are impaired in TILs and how such impairment is controlled by the established mediators of tumor-mediated immune suppression.

PD-1 is a critical mediator of tumor-mediated immune suppression and it is increased in abundance on the surface of T cells in response to continuous antigen exposure. In persistent viral infections, PD-1 signaling maintains an exhausted phenotype among CD8<sup>+</sup> T cells (9). PD-1 engagement has multiple effects on T cell activation. These include activation of phosphatase and tensin homolog (PTEN) (10), recruitment of the phosphatase sarcoma homology 2 domain phosphatase 2 (SHP-2) to the interface between CTLs and their target tumor cells (11, 12), suppression of the sustained activation of Akt and Ras pathways with consequences for cell cycle regulation (13), inhibition of glycolysis and the promotion of lipolysis and oxidative metabolism (14), and the increased abundance of the proapoptotic protein Bim (15). PD-1 engagement inhibits the T cell stop signal upon contact with an antigen presenting cell (APC) (16) and the formation of stable cell couples (17). However, it remains unresolved as to whether and how PD-1 signaling disrupts the cellular events required for CTL-mediated target cell killing.

The killing of target cells by CTLs requires carefully orchestrated steps of cellular reorganization, a process known as polarization. CTLs must bind to the target cells, use actin polymerization to stabilize the cellular interface (known as the immune synapse), relocate the microtubule organizing center (MTOC) from behind the nucleus to the center of the cellular interface, and finally release the contents of cytolytic granules directed toward the target cell (18, 19). The increase in the T cell cytoplasmic calcium ion (Ca<sup>2+</sup>) concentration,

as mediated by store-operated calcium entry using the endoplasmic reticulum (ER) calcium sensor stromal interaction molecule (STIM) and the plasma membrane calcium channel Orai, is a key signaling step required for cytolytic granule release (20). The increase in intracellular  $\text{Ca}^{2+}$  concentration in TILs becomes diminished, likely driven by defects in proximal T cell receptor (TCR) signal transduction. Demonstrating the importance of  $\text{Ca}^{2+}$  signaling for CTL function and tumor control, loss of or mutations in STIM and Orai impair cytolytic killing and immune control of tumors, and the activation of  $\text{Ca}^{2+}$  channels in  $\text{CD8}^+$  T cells can restrain tumor growth (21-25). The increase in the T cell cytoplasmic  $\text{Ca}^{2+}$  concentration and actin dynamics are bi-directionally linked (26).

To investigate how T cell polarization and related signaling are altered when TILs interact with their target tumor cells, we adapted a well-established model of antigen-driven tumor recognition to live-cell imaging approaches *ex vivo*. A renal carcinoma cell line (Renca) expressing the influenza virus haemagglutinin (HA) as a neo-antigen (RencaHA) is recognized by  $\text{CD8}^+$  T cells from Clone 4 TCR transgenic mice (27). When adoptively transferred into a RencaHA-tumor bearing BALB/c mouse, naïve Clone 4 T cells are primed within the tumor-draining lymph nodes, infiltrate the tumor, and acquire a suppressed phenotype that is characterized by diminished cytokine secretion, decreased killing ability, and enhanced inhibitory receptor expression (28). Here, we adoptively transferred *in vitro*-activated Clone 4  $\text{CD8}^+$  T cells into RencaHA tumor-bearing mice. These transduced Clone 4 T cells homed to the tumor and acquired a suppressed phenotype. They were re-isolated for an *ex vivo* investigation of their interaction with RencaHA tumor cells. Clone 4 TILs displayed impaired cell couple maintenance with their tumor targets, which was associated with impaired F-actin distribution at the target cell interface and reduced  $\text{Ca}^{2+}$  signaling, resulting in impaired tumor cell killing. PD-1 expression on Clone 4 TILs was enhanced. Direct interaction of Clone 4 T cells with RencaHA tumor cell spheroids *in vitro* induced a similar phenotype. Blocking PD-1 engagement *in vivo* in the tumor microenvironment, but not acutely *in vitro*, enhanced tumor clearance and improved the maintenance of TIL cells. Thus, we have discovered a cellular mechanism of tumor-mediated immune suppression: Impaired maintenance of T cell polarization reduced the cytotoxicity of TILs and a polarization-impaired state was induced by the inhibitory receptor PD-1.

## Results

### The cytolytic ability of $\text{CD8}^+$ T cells is diminished as a result of tumor infiltration

To investigate the killing of tumor target cells by  $\text{CD8}^+$  TILs, we adapted a mouse model of neo-antigen  $\text{CD8}^+$  T cell tumor recognition to enable *ex vivo*, live-cell imaging approaches (Fig. 1A). RencaHA renal carcinoma cells express the haemagglutinin (HA) protein from influenza A/PR/8/H1N1 as a neo-antigen. The Clone 4 TCR recognizes the dominant H2-K<sup>d</sup>-restricted HA peptide 518-526 (IYSTVASSL). In all experiments,  $\text{CD8}^+$  T cells from Clone 4 TCR transgenic mice were activated *in vitro* with HA peptide-pulsed splenocytes and retrovirally transduced to express GFP-tagged proteins of interest. Cells were FACS sorted to ensure that sensor abundance was close to endogenous protein amounts (29, 30). Activated, transduced Clone 4  $\text{CD8}^+$  T cells were adoptively transferred into BALB/c mice

bearing established subcutaneous RencaHA tumors, and after 96 hours the GFP-positive TILs were isolated from the tumors by FACS sorting.

To explore the cytolytic capability of tumor-specific CTLs and TILs, we developed an imaging-based *ex vivo* killing assay. Renca cells plated in a glass-bottom imaging plate were pulsed with HA peptide (Renca<sup>HA</sup>) and were then overlaid with Clone 4 CTLs or TILs. We found that CTLs efficiently lysed target cells, decreasing the imaging area covered by Renca<sup>HA</sup> cells at a rate of  $8 \pm 3$  %/hour (Fig. 1, B to D). Similarly, Clone 4 CTLs specifically lysed BALB/c splenocytes pulsed with HA peptide *in vivo* (fig. S1). Non-HA peptide-pulsed Renca cells continued to proliferate, which confirmed that lysis was dependent on cognate antigen recognition. In contrast, Clone 4 TILs were poorly cytolytic, as expected (39, 45, 56). The area covered by Renca<sup>HA</sup> cells was cleared by Clone 4 TILs at a significantly reduced rate of  $3 \pm 1$  %/h (Fig. 1, B to D).

### MTOC polarization in TILs and cell couple maintenance is impaired

In CTLs, relocation of the MTOC from behind the nucleus to the target cell interface after cell coupling is critical for lytic granule release and cytolytic activity (31). To visualize CD8<sup>+</sup> T cell MTOC polarization toward the Renca target cell interface, tubulin-GFP-expressing, HA-specific Clone 4 CTLs and TILs were imaged during their interaction with Renca<sup>HA</sup> cells by spinning disk confocal microscopy. Whereas all CTLs relocated their MTOC to the cellular interface within 7 min, fewer TILs quickly relocated their MTOC towards the tumor cell contact site (Fig. 2, A to C, fig. S2 and Movie S1).

We also investigated Clone 4 CD8<sup>+</sup> T cell–target cell coupling and morphology and found that TILs formed tumor cell couples less frequently than did CTLs: Whereas the initial contact of Clone 4 TILs with Renca<sup>HA</sup> target cells led to the formation of tight cell couples in  $31 \pm 6$  % of contacts,  $78 \pm 3$  % of Clone 4 CTL–tumor cell contacts led to the formation of tight couples. Clone 4 CTLs formed symmetrical, stable interfaces with Renca<sup>HA</sup> target cells throughout 12 min of observation (Fig. 2D). Initial immune synapses between Clone 4 TILs and Renca<sup>HA</sup> target cells were also symmetrical, but polarization was diminished starting as rapidly as seconds after tight cell coupling (Fig. 2E). To quantify this difference in Clone 4 CD8<sup>+</sup> T cell polarization, we determined the occurrence of lamellae pointing away from the cellular interface (Fig. 2E, red arrows). T cells continuously extend large lamellae, but whereas directing such lamellae towards the interface is expected to be stabilizing, directing them away from the interface (“off-synapse lamellae”) may weaken the interface (32, 33). Off-synapse lamellae occurred in a higher proportion of TILs than of CTLs (Fig. 2F) and were observed earlier during cell coupling (Fig. 2G). Furthermore, TILs remained at the initial site of target cell binding less efficiently:  $14 \pm 3$  % of TILs translocated across the target cell surface by at least one interface diameter from the initial site of target cell binding, whereas only  $4 \pm 2$  % of CTLs exhibited translocation (Fig. 2H), as was expected based on previous studies (34, 35). Thus, our data suggest that although HA-specific TILs effectively establish cell couples with peptide-pulsed Renca target cells, they do not maintain cell couples well.

## Peripheral F-actin ring formation is destabilized in TILs compared to that in CTLs

The immune synapse is stabilized by an F-actin ring around its periphery. In cytolytic killing, clearance of F-actin from the interface center can be associated with effective secretion of cytolytic granules (36), but not always (37, 38). To determine whether impaired F-actin distributions could underpin the impaired maintenance of TIL–target cell couples, we visualized F-actin with an F-actin-binding peptide conjugated to GFP: F-tractin-GFP (39). Clone 4 CTLs and TILs expressing F-tractin-GFP were imaged (Fig. 3, A to C), and the data were automatically shape-normalized and computationally analyzed (Fig. 3, D and E, fig. S3A and Movie S2). To investigate F-actin clearance from the interface center, we measured the ratio of F-actin accumulation at the interface center relative to the entire cell (Fig. 3D). At the time of the first contact between Clone 4 T cells and Renca<sup>HA</sup> target cells, F-actin was comparably enriched in CTLs and TILs. However, F-actin enrichment at the interface center rapidly decreased in Clone 4 CTLs to 1.3-fold or less over the next 2 min, indicative of central clearing. In contrast, F-actin enrichment remained greater than 1.5-fold in Clone 4 TILs. Addressing F-actin distributions cell-wide, we measured enrichment in the 10% of the cell volume with the greatest amounts of F-tractin, which largely corresponded to the cellular interface with an emphasis on its edge (Fig. 3D). Enrichment of F-actin was increased in antigen-specific TILs relative to that in CTLs (Fig. 3E). However, TILs displayed substantially reduced F-actin amounts overall, as determined by phalloidin staining (fig. S3, B and C). Thus, the increased relative interface enrichment in Clone 4 TILs was unlikely to correspond to greater F-actin densities, which suggests that the dominant F-actin defect in Clone 4 TILs was impaired F-actin clearance from the interface center. This defect just preceded the time of occurrence of off-interface lamellae (Fig. 2G) and the time at which MTOC polarization began to fail (Fig. 2C).

To investigate whether impaired cell couple maintenance could result in loss of cytolytic effector function, Clone 4 CTLs were treated with a low concentration of Jasplakinolide, a macrocyclic peptide that promotes actin filament nucleation. At this concentration of Jasplakinolide, cell coupling is not substantially impaired but F-actin turnover is slowed (40, 41). Interface F-actin accumulation was slightly diminished after treatment with Jasplakinolide, because the 10% cell volume with the greatest F-tractin amounts were not exclusively focused on the interface anymore, but included regions at the T cell distal pole (Fig. 3E). Treatment with Jasplakinolide significantly reduced the rate of target cell lysis by CTLs to that displayed by TILs (Fig. 3F). Treatment of Clone 4 CTLs with a higher concentration of Jasplakinolide that impairs cell coupling (41) further reduced killing (Fig. 3F). The similar phenotypes of Jasplakinolide-treated Clone 4 CTLs and untreated TILs suggest that tumor-induced disruption of actin remodeling may suppress the lytic activity of TILs.

## Interface recruitment of negative regulators of F-actin is more extensive in TILs than in CTLs

T cell actin remodeling is a dynamic process, which is promoted by the Arp2/3 complex. Coronin 1A and cofilin inhibit the Arp2/3 complex and promote the severing and depolymerization of F-actin, respectively. Actin regulators need to localize to regions of T cell F-actin turnover to be effective (30). To investigate mechanisms of altered F-actin

dynamics, we imaged Clone 4 CTLs and TILs that were transduced to express cofilin-GFP, Arp3-GFP, or Coronin 1A-GFP (Fig. 4, A to G; fig. S4, A to G; and Movies S3 to S5). Although we found no difference in the relative amounts of Arp3 recruited to the interface when we compared Clone 4 CTLs and TILs, the amounts of cofilin and Coronin 1A that were recruited were greater in Clone 4 TILs within seconds of tight cell coupling than in Clone 4 CTLs (Fig. 4F). These differences in cofilin and Coronin 1A interface enrichment persisted after prolonged tight cell coupling (Fig. 4G). The distribution of the cofilin-activating phosphatase chronophin (42) was similar to that of cofilin itself (fig. S4, H to K, and Movie S6). To establish whether the increase in amount of cofilin recruited to the interface was associated with enhanced cofilin activation, we determined the relative amounts of active non-phosphorylated cofilin by phos-tag Western blotting analysis of Clone 4 CTL and TIL lysates activated on plates coated with antibodies against CD3. At all times, the percentage of active, unphosphorylated cofilin was increased within TILs when compared to that in CTLs (Fig. 4, H and I, and fig. S5A). However, the overall abundance of cofilin was reduced in Clone 4 TILs when compared to that in CTLs, as determined by Western blotting analysis (fig. S5B). The increased interface retention of cofilin and Coronin 1A in TILs after cell coupling suggests that impaired F-actin stability in TILs could underpin the diminished F-actin translocation to the edge of the interface (Fig. 3D) and the accompanying defect in cell couple maintenance (Fig. 2, D and E) that we observed earlier.

### Activation-induced cytoplasmic $\text{Ca}^{2+}$ influx is impaired in TILs compared to that in CTLs

To identify signaling defects associated with TIL polarization impairment, we also investigated the T cell cytoplasmic  $\text{Ca}^{2+}$  concentration in Clone 4 T cells after activation by  $\text{Renca}^{\text{HA}}$  cells with the  $\text{Ca}^{2+}$ -sensitive, ratiometric fluorescent dye Fura-2 (Fig. 5 A and B, and Movie S7). In Clone 4 CTLs, the cytoplasmic  $\text{Ca}^{2+}$  concentration increased rapidly after initial target cell contact, often after formation of a first small membrane contact. In contrast, after activation, the cytoplasmic  $\text{Ca}^{2+}$  concentration increase in Clone 4 TILs was delayed sometimes up to minutes and was reduced in magnitude (Fig. 5, C and D), as expected (21, 24, 25). To determine whether the  $\text{Ca}^{2+}$  signaling defect could be linked to impaired killing and cell couple maintenance, we treated HA-specific CTLs with  $\text{Ni}^{2+}$  to block plasma membrane calcium channels and 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA) to buffer cytoplasmic  $\text{Ca}^{2+}$ .  $\text{Renca}^{\text{HA}}$  target cells exhibited  $5.8 \pm 0.3\%$  area shrinkage/hour without treatment in our imaging-based killing assay and  $2.9 \pm 1.0\%$  after treatment with  $\text{Ni}^{2+}$  and BAPTA (Fig. 5E). The frequency of tight cell couple formation between Clone 4 TILs and  $\text{Renca}^{\text{HA}}$  target cells was reduced from  $78 \pm 3\%$  without treatment to  $34 \pm 7\%$  after treatment with  $\text{Ni}^{2+}$  and BAPTA (Fig. 5F). Whereas  $80 \pm 7\%$  of CTLs treated with  $\text{Ni}^{2+}$  and BAPTA produced off-synapse lamellae, only  $24 \pm 4\%$  of untreated CTLs exhibited the same behavior (Fig. 5G) although the onset time did not differ substantially (Fig. 5H). Similarly,  $29 \pm 8\%$  of CTLs treated with  $\text{Ni}^{2+}$  and BAPTA translocated over the target cell surface by at least a full interface diameter, but only  $4 \pm 2\%$  of untreated CTLs translocated over the same distance (Fig. 5I). These data suggest that diminished  $\text{Ca}^{2+}$  signaling could contribute to impaired cell couple maintenance in TILs (Fig. 5J).

## Direct interaction between CTLs and tumor cell spheroids induces a polarization-impaired state

To determine whether infiltration of CTLs into three-dimensional (3D) tumor cell structures was sufficient to impair the polarization of CD8<sup>+</sup> T cells, we grew Renca cells as spheroids in Matrigel. Overnight interaction of Clone 4 CTLs with spheroids incubated with HA peptide (Fig. 1A) resulted in Clone 4 cell infiltration to generate spheroid-infiltrating lymphocytes (SILs) (Fig. 6A). After we re-isolated these cells from the spheroids, we found that the rate of killing of Renca<sup>HA</sup> target cells was reduced from  $5.6 \pm 0.7\%$  area shrinkage/hour after co-culture with activated CTLs to  $3.5 \pm 0.9\%$  after co-culture with SILs (Fig. 6B). PD-1 abundance was increased in SILs when compared to that in CTLs (Fig. 6C). Additionally, the frequency of the formation of tight cell couples after initial SIL contact with Renca<sup>HA</sup> target cells was reduced when compared to that of Clone 4 CTLs from  $78 \pm 3\%$  to  $47 \pm 4\%$  ( $P = 0.007$ ), and the maintenance of SIL couples with Renca<sup>HA</sup> cells was impaired (Fig. 6, D to F). Finally, the cytoplasmic Ca<sup>2+</sup> concentration was substantially reduced after activation of SILs compared to that in CTLs (Fig. 6G). Thus, infiltration of Clone 4 CTLs into Renca<sup>HA</sup> cell spheroids induced a polarization-impaired state that shared critical features with that of functionally impaired TILs.

## Blocking PD-1 in vivo enhances TIL cytotoxicity and tumor rejection

To identify potential in vivo regulators of the diminished maintenance of TIL cytoskeletal polarization, we investigated the role of PD-1. The expression of PD-1 limits anti-tumor immunity (1, 3), and we confirmed that the abundance of PD-1 was increased on TILs and endogenous CD8<sup>+</sup> T cells from RencaHA tumors when compared to that on activated CTLs (Fig. 7A). When RencaHA tumor-bearing mice were treated with a blocking monoclonal antibody (mAb) against PD-1 for 6 days before and after the adoptive transfer of Clone 4 CTLs (Fig. 7B), tumor growth was significantly reduced after antibody treatment when compared to that in control treated mice (Fig. 7C), as was expected based on previous findings (3). In addition, killing of Renca<sup>HA</sup> tumor cells *ex vivo* by Clone 4 TILs isolated from anti-PD-1-treated mice was substantially enhanced compared to that by Clone 4 TILs isolated from untreated control mice (Fig. 7D).

PD-1 inhibitory signaling is activated after engagement by its ligand programmed death-ligand 1 (PD-L1), which is expressed on tumor cells, tumor-associated immune cells, or both (1). To investigate the functional relevance of tumor-expressed PD-L1, we generated a RencaHA-PD-L1<sup>-/-</sup> cell line (fig. S6A). Loss of PD-L1 expression induced spontaneous tumor regression in 4 out of 10 inoculated mice and increased survival (fig. S6, B and C). In combination with the adoptive transfer of HA-specific Clone 4 CTLs, all mice bearing RencaHA-PD-L1<sup>-/-</sup> tumors underwent complete tumor clearance, compared to only 1 of 7 control mice (Fig. 7, E and F). Thus, these data suggest that PD-L1 on RencaHA cells contributes to the suppression of adoptively transferred Clone 4 CTLs. Together, the experiments with PD-1 blocking antibodies and PD-L1-deficient tumor cells indicate that PD-1 is a critical element of in vivo tumor immune suppression in the RencaHA model.

### PD-1 promotes the impaired maintenance of TIL polarization in vivo

To investigate whether blockade of PD-1 engagement within the tumor microenvironment restored TIL polarization, we studied TILs isolated from RencaHA tumor-bearing mice treated with antibody against PD-1 or from RencaHA-PD-L1<sup>-/-</sup> tumor-bearing mice and found that  $9 \pm 5\%$  and  $11 \pm 5\%$ , respectively, displayed translocation of at least one interface diameter over the target cell surface after immune synapse formation as compared to  $14 \pm 3\%$  of TILs treated with buffer alone (fig. S7A). The production of off-synapse lamellae was not reduced by PD-1 blockade (fig. S7, B and C). In TILs from mice treated with antibodies against PD-1 and in TILs from RencaHA-PD-L1<sup>-/-</sup> tumor-bearing mice, F-actin clearing from the interface center was significantly enhanced at multiple times and resembled that of Clone 4 CTLs (Fig. 8A and fig. S7D). Similarly, cofilin accumulation at the interface was not enhanced in Clone 4 TILs from mice treated with antibody against PD-1 or in Clone 4 TILs isolated from mice with RencaHA-PD-L1<sup>-/-</sup> tumors (Fig. 8, B and C, and fig. S7E). Together, these data suggest that PD-1 engagement impairs cytoskeletal rearrangement in TILs in vivo and thus may contribute to their diminished cytolytic function.

### Acute in vitro blockade of PD-1 does not restore cytoskeletal polarization or killing

To further understand the role of PD-1, we investigated the effects of acute PD-1 blockade performed only during in vitro killing and imaging assays. When HA-specific Clone 4 TILs or CTLs were treated with the same antibody against PD-1 as that used in vivo or when RencaHA-PD-L1<sup>-/-</sup> target cells were used, target cell killing was not enhanced (Fig. 9A). Similarly, the lytic activity of HA-specific TILs or CTLs was not inhibited by RencaHA target cells that overexpressed PD-L1 (Fig. 9A and fig. S8A). These data suggest that PD-1 does not regulate cytolysis by TILs and CTLs in vitro in an acute manner.

When we investigated the effect of acute PD-1 blockade on F-actin distribution and Ca<sup>2+</sup> influx in HA-specific CTLs and TILs, we found that it did not enhance these critical elements of cellular polarization (Fig. 9, B to D, and fig. S8, B to D). On the contrary, acute treatment with antibody against PD-1 increased the percentage of Clone 4 CTL and TIL cell couples with Renca<sup>HA</sup> target cells that translocated from their initial target cell binding site by at least one interface diameter increased from  $4 \pm 2\%$  to  $24 \pm 7\%$  for CTLs and from  $14 \pm 3\%$  to  $50 \pm 9\%$  for TILs (Fig. 9B). However, acute PD-1 blockade did not affect F-actin central clearing (Fig. 9C) nor enrichment in the 10% of the cell volume with the highest F-tractin amounts (fig. S8D). Acute PD-1 blockade also did not enhance Ca<sup>2+</sup> signaling in Clone 4 CTLs, but indeed impaired it around the time of tight cell coupling (Fig. 9D). Whereas PD-1 blockade in vivo thus restored a substantial fraction of the impaired cell couple maintenance of TILs, acute in vitro blockade could not do so. On the contrary, PD-1 blockade impaired some elements of CTL signaling and cell polarity consistent with in vitro data in a model of persistent viral infection (17).

## Discussion

The mechanisms underpinning the defective killing ability of TILs are still uncertain. A number of alterations in TIL activation have been described previously, including reduced actin polymerization (43-45), reduced Ca<sup>2+</sup> signaling among other proximal defects (24, 46,



47), increased expression of the inhibitory signaling mediators phosphatase sarcoma homology 2 domain phosphatase 1 (SHP-1) and cytokine-inducible SH2-containing protein (Cish) (46, 48), and decreased functional avidity of the TCR (49). In human TILs activated by super-antigen–incubated B cell lines, MTOC recruitment to the cellular interface and F-actin clearance from its center are impaired in a Galectin-3–dependent manner (50). Metabolic defects in the glucose-poor tumor microenvironment are likely to occur because the enhancement of glycolysis in TILs leads to improved tumor killing (51, 52). By establishing an experimental system for the rapid *ex vivo* imaging of the antigen-driven interaction of TILs with their target tumor cells, we further described the cellular mechanism of immune suppression by tumor cells: Prolonged PD-1 signaling in vivo impaired Ca<sup>2+</sup>-sensitive cytoskeletal polarization and diminished the lytic activity of TILs. We recapitulated key features of TIL suppression using the interaction of only CTLs and tumor cell spheroids. Time is a critical element of this mechanism because, first, the polarization defect affected cell couple maintenance more than cell couple formation and, second, PD-1 blockade was only effective over days within the tumor microenvironment, but not acutely in vitro.

Whereas TILs and SILs formed cell couples with their target tumor cells, often, within seconds of cell coupling, TILs and SILs increasingly developed lamellae pointing away from the target cell interface. Recruitment of the Arp2/3 complex to the interface periphery, the principal means by which T cells generate F-actin (53), was similar between TILs and CTLs. In contrast, recruitment of cofilin and Coronin 1A, key negative regulators of F-actin, was enhanced in TILs compared to that in CTLs. This is expected due to slower actin turnover by inhibition of the Arp2/3 complex by Coronin 1A and destabilization of F-actin filaments by cofilin. Reduced F-actin turnover is consistent with the decreased ability of TILs to move F-actin to the interface edge while clearing the center, which is considered to be critical for effective killing of target cells (36). Four elements of tumor immune suppression have already been linked to cofilin. The prostaglandin PGE<sub>2</sub> is an established element of Clone 4 tumor immunosuppression (54) and it inhibits actin polymerization during phagocytosis through the activation of cofilin (55). Similarly, adenosine receptors, which in the tumor microenvironment mediate immune suppression by adenosine (6, 7), inhibit cofilin inactivation in cardiomyocytes (56). Cofilin activation can also be inhibited by oxidation in T cells and during mesenchymal cell motility (57, 58). The low-oxygen environment of solid tumors (59) is consistent with enhanced cofilin activity. Finally, reduced amounts of intracellular ATP induce cofilin activation by chronophin in neurons (60), a process that is equally conceivable in the energy-starved tumor microenvironment. Consistent with our suggestion that coronin 1A impairs F-actin dynamics in TILs, localized coronin 1A inhibition in CD4<sup>+</sup> T cells enables the efficient formation of a signaling-enhancing, lamellar F-actin network (40).

We observed that diminished PD-1 engagement in vivo partially restored the ability of TILs to maintain a polarized cell couple with and kill their target tumor cells. In contrast, acute blockade of PD-1 during the interaction of TILs with target Renca tumor cells in vitro did not rescue either impaired TIL polarization or target cell killing. These data suggest that extended in vivo engagement of PD-1 induces a "polarization-impaired state" that may require hours to days to form and revert. In support of this hypothesis, co-culture of primary T cells with various tumor cell types for 24 hours diminishes the ability of the T cells to

form cell couples with allogeneic antigen-pulsed B cells (44), which is partially controlled by PD-1 (43). Similarly, in a model of persistent lymphocytic choriomeningitis virus (LCMV) infection, T cell mobility, an indicator of polarization, was impaired in a PD-1-dependent manner (17). Over time, T cells can switch between suppressed and active states. In vitro culture of TILs in IL-2 for more than 6 hours can overcome their diminished ability to kill target tumor cells (61), and resting of TILs for >24 hours in vitro restores impaired tetramer binding to the TCR (49). In the LCMV model, restoration of T cell motility in vivo became apparent after 1 hour of anti-PD-1 treatment and increased over the next 16 hours (17).

PD-1 could also exert indirect effects within the tumor microenvironment through enhancing the lineage stability of FoxP3<sup>+</sup> Tregs (62), suppressing IFN- $\gamma$ -dependent innate and adaptive immunity (63), and enabling the recruitment of novel peripheral T cell clonotypes to the tumor (64, 65). In addition, engagement of the costimulatory receptor CD28 is required for the restoration of T cell function by PD-1 blockade in vivo (66, 67). Whereas HA-specific Clone 4 TILs may have access to the CD28 ligands CD80 and CD86 on the surface of tumor-infiltrating professional APCs, they had no such opportunity during their interaction with Renca<sup>HA</sup> target cells in vitro. At this time, it is still unresolved as to whether the extended time of PD-1 engagement or indirect effects of PD-1 within the tumor is more important in the establishment of the polarization-impaired state of TILs. The need for extended PD-1 engagement within the context of the tumor microenvironment makes it difficult to link the established signaling roles of PD-1, which have largely been investigated acutely and in vitro, to the in vivo inhibition of TIL polarization and function by PD-1. Nevertheless, transcriptional changes in TILs are conceivable, because sustained engagement of another receptor, the TCR by antigen, can substantially alter the transcriptional program of T cells (68), as was also shown in the context of cancer (69). In this context, differential expression of cytoskeletal components as determined here may contribute to the TIL polarization defect even though the dependence of this effect on PD-1 still needs to be determined. Metabolic changes are also conceivable, because enhanced tumor cell killing by blockade of PD-1 is associated with increased glycolysis in TILs (52). Future investigations of the mechanisms of PD-1 function should take into account the difference between the sustained in vivo and acute in vitro roles that we have observed.

## Materials and Methods

### Cells and media

Complete medium consisted of RPMI-1640 with L-glutamine (Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone defined, US source, GE healthcare), 50  $\mu$ M  $\beta$ -mercaptoethanol (Gibco), and PenStrep (Gibco) at 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. RencaWT cells were maintained in complete medium. RencaHA cells were maintained in complete medium supplemented with 100  $\mu$ g/ml geneticin. Please note that "Renca<sup>HA</sup>" refers to either RencaWT or RencaHA cells incubated with a large excess of exogenous agonist peptide. "Renca<sup>WT</sup>" refers to RencaWT cells that were not incubated with peptide. Clone 4 T cells were maintained in complete medium supplemented with 50 U/mL rh-IL-2 (NIH/NCI BRB preclinical repository; referred to as "IL-2 medium").

Phoenix cells were maintained in DMEM with 4.5 g/L D-glucose, L-glutamine and sodium pyruvate (Gibco), supplemented with 10% FBS (Hyclone define, US source, GE healthcare), MEM non-essential amino acids (Gibco), PenStrep (Gibco), 300 µg/mL Hygromycin (Invitrogen), and 1 µg/mL Diphtheria toxin (Sigma). Renca WT, RencaHA, and Phoenix cell lines were tested for the absence of mycoplasma.

## Mice

Clone 4 TCR transgenic mice (RRID: IMSR\_JAX:005307) were bred and maintained at the University of Bristol under specific pathogen-free conditions. All Clone 4 mice were culled for experimental use between 6 and 12 weeks of age. BALB/c mice were purchased from Charles River Laboratories at 6 weeks of age and then maintained at the University of Bristol under specific pathogen-free conditions. All mice were culled using schedule one methods and any experimental procedures were conducted in accordance with protocols approved by the UK Home Office.

## Clone 4 CD8<sup>+</sup> T cell isolation, stimulation, and retroviral transduction

The procedures used for the isolation and stimulation of TCR transgenic T cells were extensively detailed previously (29, 70, 71). Briefly, red blood cells were removed from dissociated spleens using ACK lysis buffer (Gibco) and splenocytes were plated at  $5 \times 10^6$  cells per well, in 1 mL of complete medium, in a 24-well plate. K<sup>d</sup>HA peptide (IYSTVASSL) was added to a final concentration of 1 µg/mL, and the cells were incubated overnight. Primed cultures were washed five times in PBS to remove unbound peptide and then retrovirally transduced as extensively detailed previously (29, 70, 71). Briefly, T cells were transduced to express GFP-tagged proteins using recombinant Moloney murine leukemia virus (MMLV). Plasmids for the retroviral expression of F-tractin-GFP (71), tubulin-GFP (72), cofilin-GFP (30), coronin 1A-GFP (30) and chronophin-GFP (71) were described previously. T cells were resuspended in 2 mL of retrovirus-enriched supernatant from transfected Phoenix-E cells. Protamine sulphate was added to a final concentration of 8 µg/mL. Plates were centrifuged for 2 hours at 200g at 32°C. After transduction, the cells were re-plated at  $4 \times 10^6$  cells per well, in 2 mL of IL-2 medium, in a 24-well plate. Cells were cultured for a further two days. CTLs were sorted and imaged or were adoptively transferred into Renca<sup>HA</sup> tumor-bearing mice, on day four.

## Renca<sup>HA</sup> tumor growth in BALB/c mice and adoptive transfer of Clone 4 CD8<sup>+</sup> T cells

To induce tumor growth, 6- to 8-week old female BALB/c mice were injected subcutaneously in the scruff of the neck with  $1 \times 10^6$  Renca<sup>HA</sup> cells suspended in PBS. The mice were monitored regularly to assess tumor growth. Tumor size was measured using a caliper and calculated using the following equation: volume (V) =  $L^2 \times W/2$ , where length (L) was the longest measurement and width (W) was measured perpendicularly to length. Mice were culled if tumors exceeded 15 mm in any one direction or any humane end points were reached. GFP-positive Clone 4 CTLs were resuspended in PBS at  $2 \times 10^7$  cells/mL, and 200 µL of cells were injected into the tail vein of a tumor-bearing mouse. Unless otherwise specified, only mice bearing tumors greater than  $500 \mu\text{m}^3$  in volume were used, and tumors were harvested 96 hours after transfer.

### **In vivo anti-PD-1 immunotherapy**

Mice received three doses of either monoclonal antibody against PD-1 (RMP1-14, Bio X cell, RRID: AB\_10949053) or rat IgG2a isotype control (2A3, Bio X cell, RRID: AB\_1107769) on days one, three, and five of treatment by tail vein injection. Each dose consisted of 250 µg of mAb in 200 µL of PBS. Adoptive transfer of CTLs occurred on day two, and the mice were culled on day six. Tumor measurements were taken on days one and six before culling.

### **Isolation of TILs**

Tumors were collected into 3.2 mL of RPMI-1640, roughly chopped with sterile scissors, and tumor cell dissociation enzymes (Miltenyi Biotec) were added according to the manufacturer's instructions. Tumors were incubated at 37°C for 45 min and briefly vortexed every 10 min to enhance dissociation. The tumor suspension was then passed through a 40-µm sieve, and red blood cells were removed with ACK lysis buffer. Cells were resuspended in 600 µL of MACs buffer (PBS, 2 mM EDTA, 0.5% BSA) with 65 µL of MACs microbeads (Miltenyi) and incubated at room temperature for 30 min. CD8a (Ly-2) microbeads were used to enrich for CD8<sup>+</sup> T cells, whereas CD45 microbeads were used to enrich for all lymphocytes. Positive selection of the magnetically labelled cell populations was performed using MACS LS-columns (Miltenyi Biotec). Collected cells were resuspended in 500 µL of imaging buffer for immediate sorting or 500 µL of FACS buffer for antibody staining.

### **Generation of SILs**

RencaWT mCherry cells were resuspended at a concentration of  $1 \times 10^5$  cells/ml, mixed with Matrigel (Corning) at 4°C, seeded in a 24-well plate at a final concentration of 500 cells/blob, and left to solidify for 10 min at 37°C. Cell medium (2 ml) was added to each well and incubated at 37°C for 11 days. Each blob was washed twice in PBS and incubated for 30 min with 1 ml of Cell Recovery Solution (Corning). Spheroids were collected in a 15-ml Falcon tube and pulsed with K<sup>d</sup>HA peptide at a final concentration of 2 µg/mL for 1 hour. Pulsed spheroids were re-embedded in Matrigel together with  $5 \times 10^5$  primed Clone 4 CTLs per blob. The following day, blobs were washed twice in PBS and incubated with 1 ml of Cell Recovery Solution (Corning). Spheroids were collected, washed through a 70-µm sieve, and then disaggregated to retrieve SILs in 500 µL of imaging buffer for immediate FACS sorting.

### **Imaging of Clone 4 CTLs, TILs, and SILs**

After retroviral transduction or TIL/SIL extraction, T cell cultures were sorted to isolate GFP-positive T cells and resuspended in 'imaging buffer' (10% FBS in PBS with 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>). As target cells,  $1 \times 10^6$  RencaWT cells were pulsed with K<sup>d</sup>HA peptide at a final concentration of 2 µg/mL for 1 hour (to generate Renca<sup>+HA</sup> cells). Imaging was performed in glass-bottomed, 384-well optical imaging plates (Brooks life science systems) at 37°C with a Perkin Elmer UltraVIEW ERS 6FE confocal system attached to a Leica DM I6000 inverted epifluorescence microscope and a Yokogawa CSU22 spinning disk. A 40x oil-immersion lens (NA=1.25) with 2x2 camera binning (x and y pixel size of

0.4  $\mu\text{m}$ ) was used for all imaging experiments, unless otherwise stated. Every 20 s for 15 min, a z-stack of 21 GFP images (1  $\mu\text{m}$  z-spacing) was acquired, as well as a single, mid-plane differential interference contrast (DIC) image. Some imaging experiments involved prior treatment of T cells with 40 nM Jasplakinolide, 10  $\mu\text{g}/\text{mL}$  anti-PD-1 mAb (RMP1-14, Bio X cell, RRID: AB\_10949053), or 5 mM  $\text{NiCl}_2$  with 20  $\mu\text{M}$  BAPTA-AM (Molecular Probes). In all cases, reagents were added at the desired concentration for 30 min before imaging, and the cells were incubated at 37°C, for BAPTA-AM at 30°C. All reagents, with the exception of BAPTA-AM, were also present throughout the imaging process. To image the increase in the cytoplasmic  $\text{Ca}^{2+}$  concentration, Clone 4 T cells were incubated with 2  $\mu\text{M}$  Fura-2 AM (Molecular Probes) for 30 min at room temperature in imaging buffer and washed twice thereafter. Because of limiting cell numbers, such washing was not feasible for Clone 4 TILs. Clone 4 T cells were activated with  $\text{Renca}^{\text{+HA}}$  cells as described earlier and then every 10 s for 15 min, one bright-field image, one fluorescence image with excitation at 340 nm, and one fluorescence image with excitation at 380 nm were acquired at 37°C with a 40x oil objective (NA=1.25) on a Leica DM IRBE-based wide field system equipped with Sutter DG5 illumination and a Photometrics Coolsnap HQ2 camera. For analysis, field-averaged background fluorescence was subtracted from the fluorescence data and the ratio of the Fura-2 images upon excitation at 340 nm versus 380 nm was calculated and multiplied by 100 to fit into the 8-bit display scale. Average ratio within a circular region of interest of the dimensions of the T cell was determined over time for each T cell.

### Analysis of live-cell imaging data

Image analysis protocols have previously been described (29, 70, 71). Using Metamorph image analysis software (Molecular Devices), we identified CTLs forming conjugates with  $\text{Renca}$  target cells using the DIC reference images. Tight cell couple formation was defined as the first time point at which a maximally spread immune synapse formed, or two frames after initial cell contact, whichever occurred first. To assess CTL and TIL morphology, every DIC frame after tight cell couple formation was assessed for the presence of off-synapse lamellae, defined as transient membrane protrusions pointing away from the immune synapse, followed by retraction. For each cell couple, the initial position of the immune synapse on the  $\text{Renca}^{\text{+HA}}$  target cell was compared to the position in the final frame. If the T cell had migrated by a distance greater than the diameter of the immune synapse, this was classed as a translocation event. To analyze protein spatiotemporal distribution, we used an improved version of the method described by Roybal *et al.* (30) as implemented in version 2.8 of the open source CellOrganizer software (<http://CellOrganizer.org>) (70, 73, 74). The first improvement is referred to as synapse tracking. The previous method required annotation of the cell couple synapse position for each time point, whereas the modified method requires only annotation at the initial time point. The fluorescence image for the initial frame is segmented by thresholding using the method described by Otsu (75), and an ellipse is fitted to the segmented object containing the manually annotated synapse position (the T cell). A window of 61 by 61 around the synapse position is chosen, and a circle containing the target cell is found by Hough transformation of the DIC image (76). The coordinates of the endpoints of the synapse are found as the intersection points of the T cell ellipse and the target cell circle. This process is repeated for preceding or subsequent frames with the T cell location information in the previous frame used to find the T cell in the

current frame. The coordinates of the two synapse endpoints are saved across time. The second improvement is referred to as alignment refinement. To correct for inadvertent rotation during the initial alignment of all of the cells within the population to be analyzed, we implemented two modifications. The first step is to infer a rotational angle around the z-axis with a regression model using as features the intensities of evenly distributed coordinates in the cylinder originating from the synapse center. The second step is to decide whether to flip the cell by 180 degrees around the y-axis using a binary classifier with the total intensity of the first and second half of all slices as features. The regressor and classifier were trained with images that had been manually annotated. Finally, we fit a line through the synapse region in the central slice and perform a fine correction of the rotation around the z-axis using the angle of that line. As a first measurement of protein enrichment, we defined the 10% of the cell volume with the highest fluorescence intensity across the average of the entire population of analyzed cells and determined enrichment in this volume relative to the entire cell, as established previously (30). Additional regions of interest were defined by geometrical criteria. For the synapse region in cofilin-GFP-expressing T cells, we used a cylinder normal to and one voxel distant from the synapse plane, with its axis centered on the synapse region, a radius of 8 voxels, and a height of 4 voxels. For the region defining the interface center in F-tractin-GFP-expressing cells, we used a cylinder similar to the one in the cofilin analysis, but with a radius of 8/3 voxels. The computational analysis of cofilin-GFP distributions required additional adaptations because cofilin-GFP accumulated prominently in the nucleus. Enrichment in the 10% of the cell volume with the highest fluorescence intensity did not reliably reflect interface enrichment. Because the T cell nucleus often closely approaches the cellular interface, a cylindrical interface measurement region captured more of the interface accumulation but still not exclusively so. Therefore, we also sorted time points into those with >135% fluorescence intensity in regions of interface accumulation above cellular background and without accumulation, computationally determined enrichment in the 10% of the cell volume with the highest fluorescence intensity at only the time points with accumulation, and multiplied the enrichment intensity by the fraction of cell couples with cofilin accumulation.

### Determination of F-actin amounts by Phalloidin staining

T cells were stained with Alexa Fluor 488-conjugated phalloidin as previously described (41) and cover slips were imaged as 3D image stacks on a Leica TCS SP8 AOBS laser scanning microscope using a 40x oil NA=1.3 objective. Using ImageJ, z-stacks of phalloidin-stained T cells were maximum-projected, and these images were then 2D median-filtered using a filter radius of 1, enabling noise reduction and edge-preservation. Otsu global thresholding (75) was used to binarize the images. Because this algorithm systematically overestimated the threshold, each threshold value was multiplied by 0.2 to provide a consistent offset. Objects were detected as regions of contiguous foreground pixels (8-way connectivity); as such, holes were filled, and a watershed algorithm was used to split merged objects. Cell areas and mean fluorescent intensities were measured and cell areas were converted to volumes assuming spherical geometry.

## T cell killing assays

Renca<sup>HA</sup> or Renca<sup>WT</sup> cells were stained with 10  $\mu$ M cell trace violet (CTV) and plated at a density of  $1 \times 10^4$  cells per well of a 384-well plate (Perkin Elmer) in 100  $\mu$ L of complete medium. Cells were incubated at 37°C for a minimum of 5 hours to allow adherence and spreading. Before imaging, complete medium was exchanged for imaging buffer (50  $\mu$ L/well). Clone 4 CTLs, TILs, or SILs were resuspended in imaging buffer at a density of  $2 \times 10^5$  cells/mL. The imaging plate was then mounted on a Leica DMI6000 inverted epifluorescence widefield microscope at 37°C with a humidified CO<sub>2</sub> enrichment attachment, a motorized stage, and adaptive focus control enabled by image analysis software (Leica LAS-X). CTV stained Renca cells were imaged, and the positions for two or three fields of view were saved per well. 50  $\mu$ L of the T cell culture (giving a total of  $1 \times 10^4$  cells/well) were added per well. Images of CTV-stained Renca cells were acquired every 30 min for 10 hours, using adaptive focus control to prevent drift. Using Metamorph image analysis software, a mask was generated using thresholding for light objects and adjusted to match the fluorescent signal. The same thresholding criteria were then used for all conditions within that experiment. The area of the field covered by CTV-stained Renca<sup>HA</sup> cells was measured, the average percentage change in cell area was calculated for each time point, and a gradient describing the rate of cell area change was calculated. This was corrected for a control condition in which no T cells were added, providing a normalized value for the rate of cell area change across all experiments.

## Generation of Renca<sup>HA</sup>-PD-L1<sup>-/-</sup> and PD-L1-GFP overexpressing cell lines

The methods we used closely follow those previously described (77). We designed sgRNAs using three programs: Benchling, Broad institute, and MIT CRISPR design. The six top common hits were selected. DNA oligonucleotides encoding these sgRNAs were inserted into the pSpCas9(BB)-2A-GFP vector (Addgene). Renca<sup>HA</sup> cells were transfected with these vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours, FACS was used to separate single GFP-positive cells into the wells of a 96-well plate. Colonies were expanded and screened for PD-L1 loss by flow cytometry. Several clones were further screened to compare MHC class I expression, HA expression, and in vitro proliferative ability to the original Renca<sup>HA</sup> cell line, and the most similar clone (B10) was selected. The following primers encoding gRNA sequences were used for the CRISPR/Cas9-mediated knockout of PD-L1: pSpCas9PDL1 b F (CACCGTCCAAAGGACTTGTACGTGG) and pSpCas9PDL1 b R (AAACCCACGTACAAGTCCTTTGGAC). The cDNA encoding PD-L1 was cloned into a pSR $\alpha$ GFP vector to create a PD-L1-GFP fusion construct. Renca<sup>WT</sup> cells were then transfected with this construct using Lipofectamine 2000 and the cells were selected in medium containing geneticin (200  $\mu$ g/ml). Cells were FACS-sorted for GFP-positive cells and analyzed by staining with antibodies against PD-L1. Flow cytometry was used to assess the expression and localization of GFP-conjugated PD-L1.

## Phos-tag Western blotting to assess cofilin phosphorylation

Round-bottomed, 96-well plates were coated overnight with 10  $\mu$ g/mL anti-CD3 antibody (145-2C11, BioLegend, RRID: AB\_2632707) in PBS at 4°C. Clone 4 CTLs or TILs were

resuspended in complete medium at a concentration of  $1.5 \times 10^6$  cells/mL and incubated at 37°C. CTLs or TILs (100  $\mu$ l) were added to the well together with 100 $\mu$ l of complete medium. Cells were centrifuged in the plate for 30 s at 250g to ensure uniform contact with the anti-CD3-coated plastic. The plate was immediately incubated at 37°C for 1, 2, or 5 min. After incubation, the cells were lysed with 100  $\mu$ l of ice-cold RIPA buffer (Cell Signaling). Phos-tag reagent (Wako) was added to 15% SDS-PAGE gels according to the manufacturer's protocols. Standard ECL protocols were used for immunodetection. The antibodies used were anti-cofilin (D3F9, Cell Signaling, RRID: AB\_10622000), anti-GAPDH (14C10, Cell Signaling, RRID: AB\_10693448), and horseradish peroxidase (HRP)-conjugated anti-IgG (Cell Signaling).

### Analysis of cell surface receptor staining by flow cytometry

Cells to be fixed were stained with Zombie live/dead fixable exclusion dyes (BioLegend). Cells were incubated with Fc receptor blocking antibody (anti-CD16/CD32, clone 93, eBioscience). Surface staining was conducted in PBS containing 0.5% FBS. Cells were fixed with 4% paraformaldehyde (PFA) using standard protocols. Antibodies used for cell surface staining included: anti-CD279/PD-1 BV785 (29F.1A12, Biolegend, RRID: AB\_2563680); anti-CD274/PD-L1 BV421 (MIH5, BD Bioscience, cat no 564714); anti-CD90.1/Thy1.1 PerCP Cy5.5 (OX-7, Biolegend, RRID: AB\_961437); anti-V $\beta$ 8.1/V $\beta$ 8.2 TCR FITC (KJ16-133, eBioscience, RRID: AB\_465261). SIL PD-1 expression was normalized to that of CTLs.

### In vivo killing assay

BALB/c mice received tail vein injections of  $4 \times 10^6$  primed Clone 4 CTLs or with PBS alone. On the second day after adoptive transfer, BALB/c splenocytes were pulsed with or without K<sup>d</sup>HA peptide at a final concentration of 2  $\mu$ g/mL for 1 hour and then washed and resuspended in PBS. K<sup>d</sup>HA-pulsed splenocytes were then stained with 5  $\mu$ M CellTrace violet (CTV) (high), whereas unpulsed splenocytes were stained with 0.5  $\mu$ M CTV (low). Splenocytes were washed and resuspended in PBS. Immediately before injection, high- and low-stained splenocytes were mixed in equal numbers, and  $5 \times 10^6$  cells were injected into the BALB/c mice. After 24 hours, the splenocytes were analyzed by flow cytometry and gated on live/dead dye exclusion and CTV staining to compare numbers of CTV<sup>high</sup> K<sup>d</sup>HA-pulsed splenocytes and CTV<sup>low</sup> control splenocytes.

### Statistical analysis

Data were checked for conformity to the Normal Distribution using the SAS procedure UNIVARIATE, including the Anderson-Darling and Shapiro-Wilk tests and graphical methods. To achieve conformity to the Normal Distribution, subcellular distribution enrichment ratios had to be transformed to logarithms. Data from independent experiments were pooled if the 95% confidence intervals of the independent experiments overlapped. To confirm the validity of such pooling of data from independent experiments, sources of variation were analyzed in more detail in three representative data sets (fig. S9). Specifically, our analysis asked how significant differences were between experimental repeats within a treatment, such as CTL experimental repeat 1 versus 2 versus however many repeats there were, as opposed to differences between treatments, CTLs versus TILs. First, each data set



was tested for auto-correlation between repeated measurements on the same cell. First-order auto-correlation ( $P < 0.05$ ) was detected in all sets of repeated measurements by the Durbin-Watson statistic (SAS procedure AUTOREG). Accordingly, further analysis was conducted by the method of General Linear Models with an AR(1) covariance matrix in SAS procedure MIXED using REML estimation. The results showed that, whereas the difference between treatments was always significant, experiments within treatments did not differ significantly. We applied this analysis to three representative experiments. The SAS/STAT14.3 package of the SAS Proprietary Software, version 9.4 was used. In the determination of time until the first off-interface lamella (Fig. 2G), differences between CTLs and TILs were significant with  $P = 0.0035$ ; differences between experimental repeats had a  $P$  value of 0.06. In the determination of cofilin interface enrichment (fig. S4B), differences between CTLs and TILs were significant with  $P = 0.0015$ ; differences between experimental repeats had a  $P$  value of 0.67. In the determination of the increase in the cytoplasmic  $\text{Ca}^{2+}$  concentration, differences between CTLs and TILs were significant with  $P < 0.0001$ ; differences between experimental repeats had a  $P$  value of 0.99. Differences between experimental conditions were then analyzed at individual times. The Bonferroni correction for multiple comparisons was applied so that, for example across 11 time points analyzed, only differences with  $P < 0.0045$  were considered to be significant and are reported as such. Applying this stringent correction increased the rate of false negatives. Our quantification of protein distributions, however, involved repeated measurements that are not necessarily independent, so the over-conservatism of the Bonferroni adjustment provides a compensatory safeguard. Imaging data grouped over time ranges were not affected by this problem. The frequency of occurrence of off-synapse lamellae or cellular translocation was analyzed using the  $z$  transformation for proportions. All other data types were analyzed by Student's  $t$  tests, unpaired, unpaired with Welch's correction for unequal variance, multiple  $t$ -tests with two-stage set-up method of Benjamini, Krieger and Yekutielli, ANOVA, or by nonparametric tests, Mann Whitney  $u$ -tests, or Kruskal-Wallis test, as appropriate and indicated in the figure legends. In few cases where it was difficult to satisfy all assumptions for the statistical tests under consideration,  $P$  values were calculated using the most appropriate tests, and the test resulting in the highest  $P$  value was reported as the most conservative approach. Standard tests were executed with GraphPad Prism version 7.0e.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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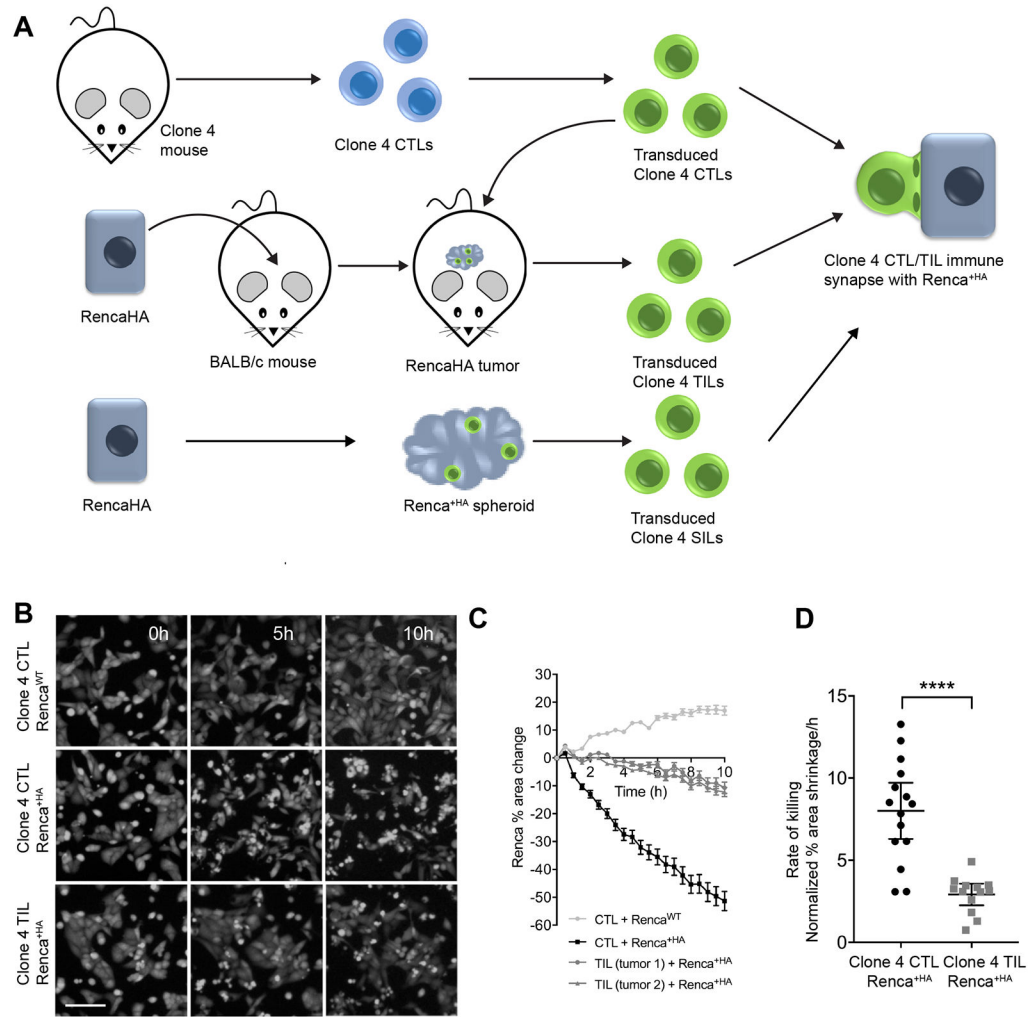
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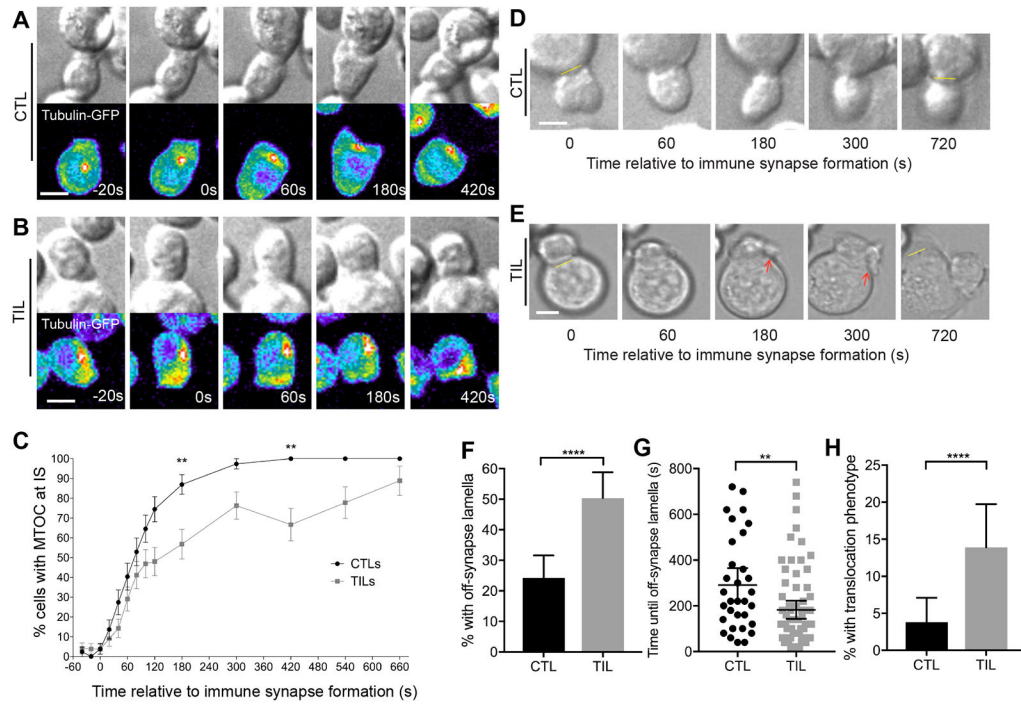
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**Fig. 1. Tumor exposure impairs the killing ability of CD8<sup>+</sup> T cells.**

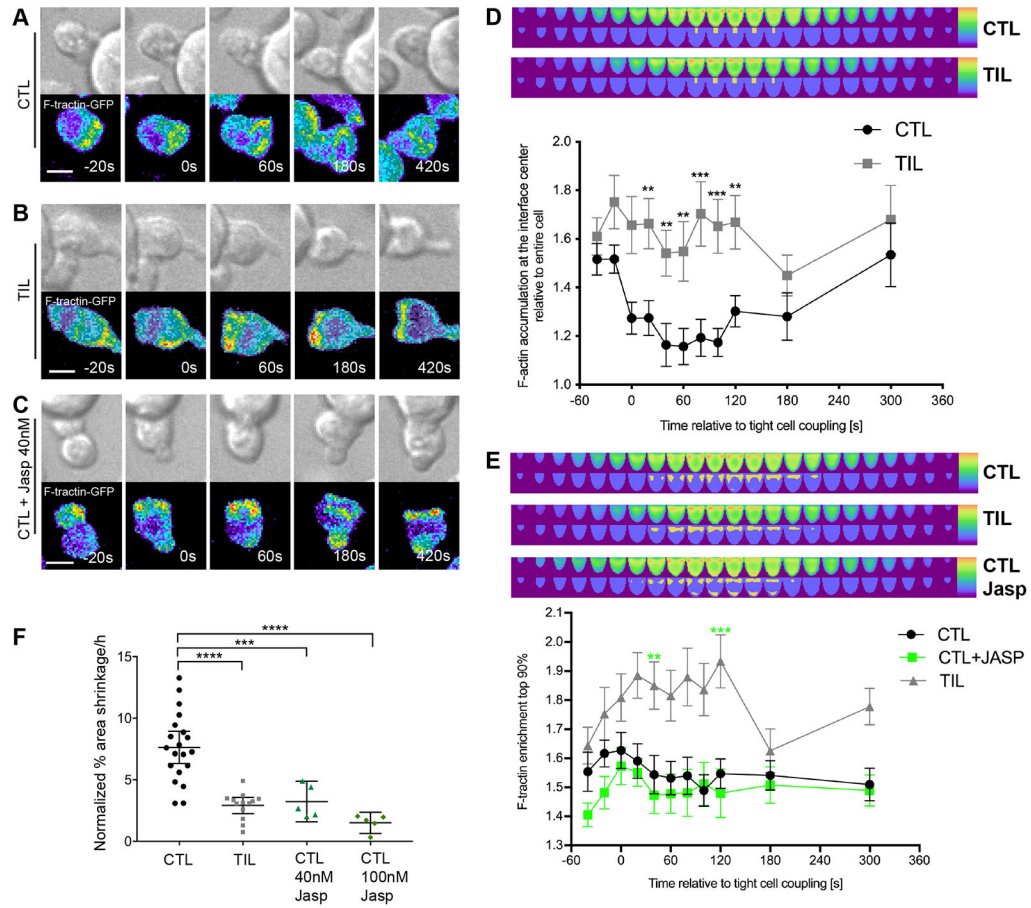
(A) Experimental schematic for imaging the interaction of HA peptide-pulsed Renca tumor cells (Renca<sup>HA</sup>) with retrovirally transduced, HA-specific, in vitro-activated CTLs in comparison to the same cells after adoptive transfer into mice with established Renca<sup>HA</sup> cells tumors or co-culture with RencaHA spheroids. (B to D) Fluorescence microscopy analysis of the killing of CTV-labelled Renca or Renca<sup>HA</sup> cells in vitro by Clone 4 CTLs or TILs. (B) Images are representative of 13 experiments. Scale bar, 100  $\mu$ m. (C) The percentage changes in the area of Renca cells are means  $\pm$  SEM from a minimum of three fields of view/time and are representative of seven experiments. (D) Average Renca<sup>HA</sup> cell death rates are means  $\pm$  95% confidence interval from seven independent experiments. \*\*\*\**P* < 0.0001 by Student's *t* test with Welch's correction.



**Fig. 2. Compared to CTLs, TILs have delayed MTOC polarization and diminished cell couple stability.**

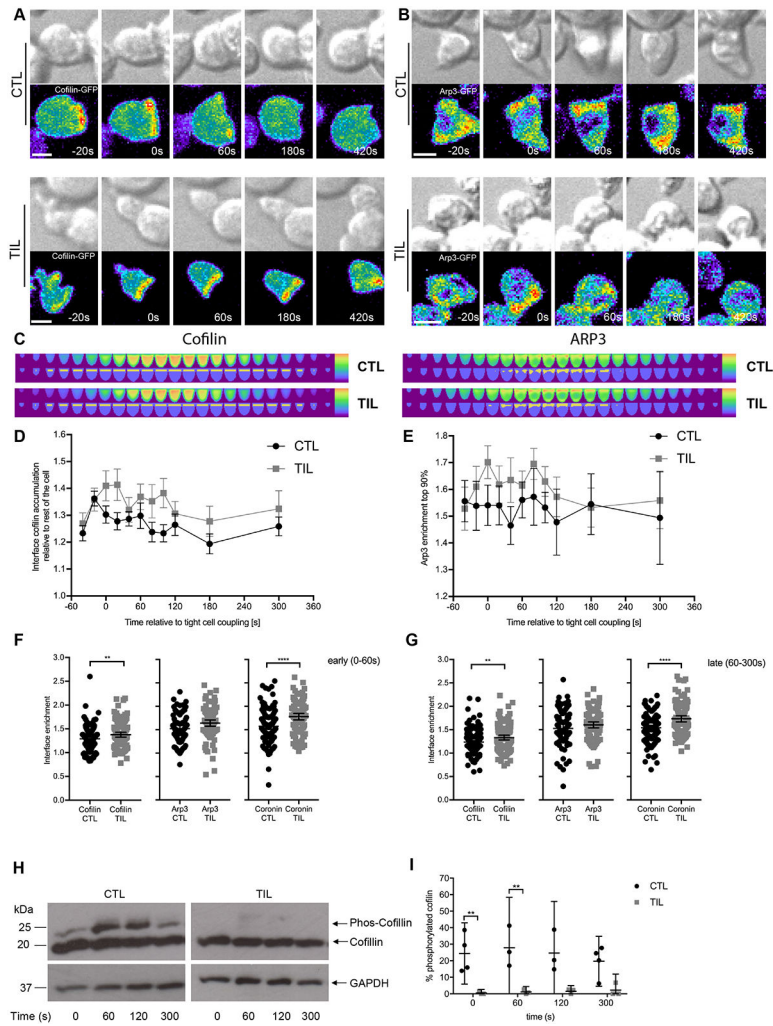
(A to C) Microscopy analysis of tubulin-GFP-expressing, HA-specific CTLs (A) and TILs (B) interacting with Renca<sup>HA</sup> cells over time. (A and B) DIC (top) and false-colored fluorescence intensity (bottom) images are representative of at least three independent experiments. (C) The percentage of cells with their MTOC localized at the immune synapse (IS) are means  $\pm$  SEM of 51 CTLs and 55 TILs from all experiments. (D to H) Microscopy analysis of HA-specific CTLs and TILs interacting with Renca<sup>HA</sup> cells over time. (D and E) DIC images with the original position of tight cell couple formation with Renca<sup>HA</sup> cells (yellow lines) and off-synapse lamellae (red arrows) are representative of at least six independent experiments. The percentages of cells with off-synapse lamellae (F), time of the first appearance of off-synapse lamella (G), and the percentage of cells that translocated away from the contact site (H) are means  $\pm$  95% confidence interval of 138 CTLs and 137 TILs from all experiments. Scale bars, 5  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$  (G), \*\* $P < 0.0036$  (C, to account for Bonferroni correction), \*\*\*\* $P < 0.0001$  by proportions z-test (C, F and H), or Mann Whitney u-test (G).





**Fig. 3. Impaired F-actin distributions at the immune synapse are associated with poor TIL killing ability.**

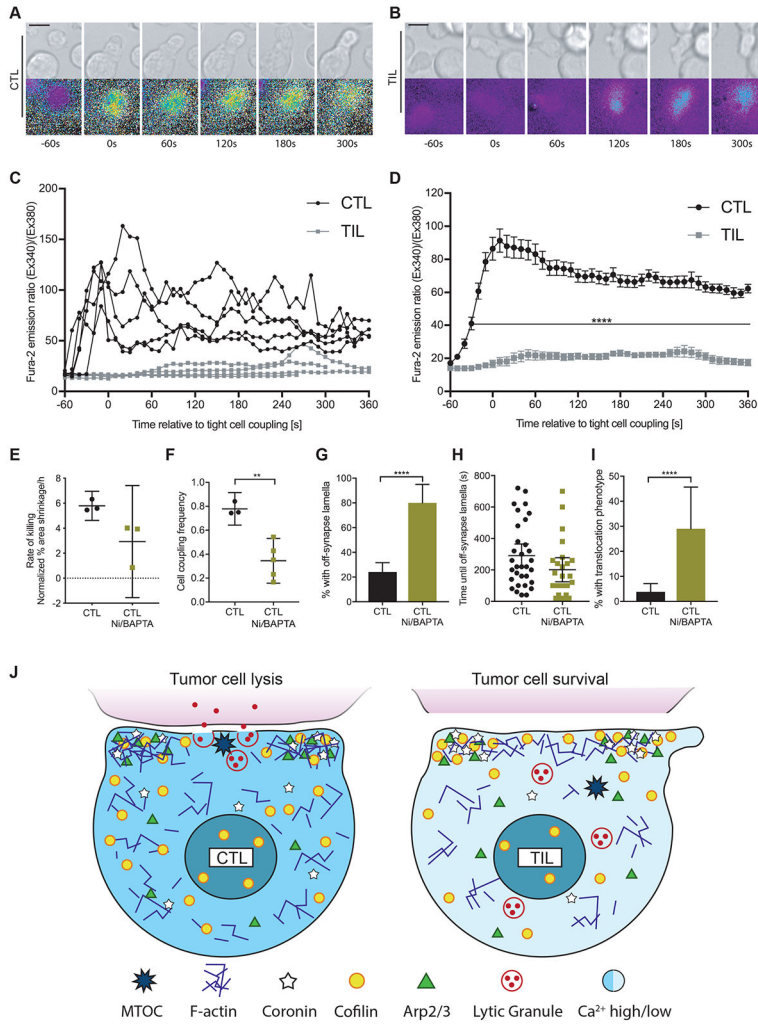
(A to E) Microscopy analysis of F-tractin-GFP-expressing Clone 4 CTLs, with and without treatment with 40 nM Jasplakinolide, and TILs interacting with Renca<sup>HA</sup> cells over time. (A to C) DIC (top) and false-colored fluorescence intensity (bottom) images are representative of at least two independent experiments. (D and E) Computational analysis of GFP fluorescence distribution is displayed in horizontal slices perpendicular to the immune synapse averaged over all cells and times with the location of the central cylinder (D) and the 10% of the cell volume with the most intense F-actin accumulation (E) highlighted in yellow. Quantification of F-actin enrichment in TILs and CTLs (D) or in TILs and CTLs treated with and without Jasplakinolide (Jasp) (E) in the highlighted volumes are means  $\pm$  SEM of 60 CTLs, 51 TILs, or 40 Jasp-treated CTLs from at least three independent experiments. (F) Fluorescence microscopy analysis of the killing of Renca<sup>HA</sup> cells in vitro by HA-specific CTLs treated with or without Jasp and by TILs. Average Renca<sup>HA</sup> cell death rate data are means  $\pm$  SEM from at least four independent experiments. Scale bars, 5  $\mu$ m. \*\* $P$  < 0.0045 (D and E, to account for Bonferroni correction), \*\*\* $P$  < 0.001, and \*\*\*\* $P$  < 0.0001 by Student's t-test (D), one-way ANOVA (E), or pair-wise Student's t-test with Welch's correction (F).



**Fig. 4. Cofilin and coronin, but not Arp2/3, are more extensively enriched at the cell contact interface in TILs.**

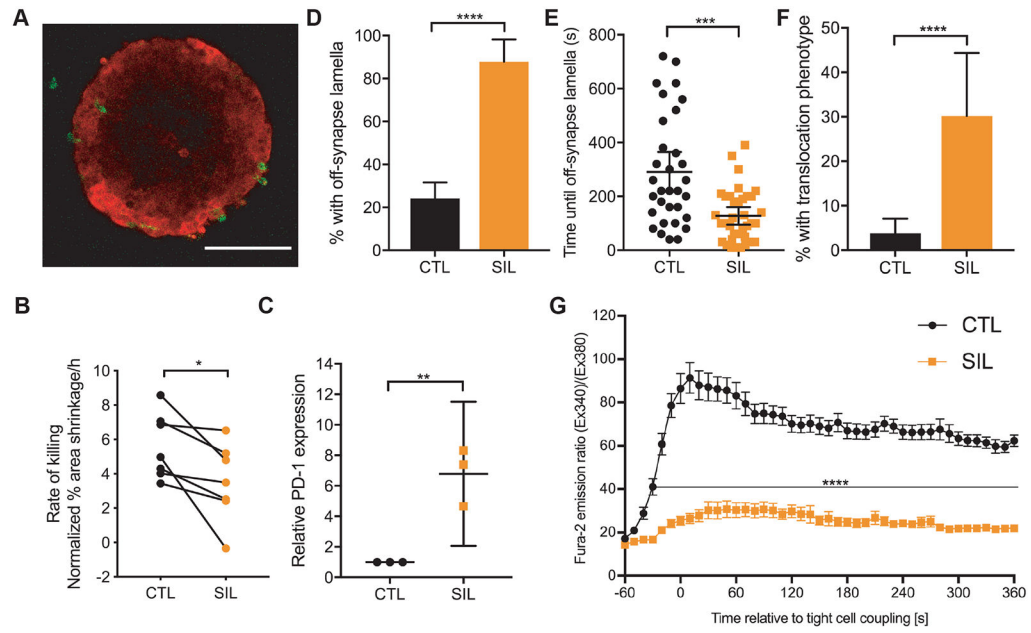
(A to G) Microscopy analysis of cofilin-GFP-expressing (A) or Arp3-GFP-expressing (B) HA-specific CTLs and TILs interacting with Renca<sup>HA</sup> cells over time. DIC (top) and false-colored fluorescence intensity (bottom) images are representative of at least two independent experiments. (C) Computational analysis of GFP fluorescence distribution is displayed in horizontal slices perpendicular to the immune synapse averaged over all cells and time points with the interface region cylinder (cofilin) and the 10% of the cell volume with the most intense Arp3 accumulation highlighted. (D to G) Quantification of interface GFP enrichment at individual times (D and E) and at early (F) and late (G) times in the highlighted volumes are means  $\pm$  95% confidence interval of 90 CTLs and 58 TILs (D) or 56 CTLs and 51 TILs (E) from all experiments with the addition of coronin 1A data (fig. S4, D to G) in (F) and (G). (H and I) Western blotting analysis of phosphorylated cofilin (Phos-Cofilin) in the lysates of HA-specific CTLs and TILs after activation with antibodies against CD3 for the indicated times. Blots (H) are representative of at least three independent experiments (see also fig. S5A). (I) The relative amounts of Phos-Cofilin are expressed as a percentage of that of total cofilin and are means  $\pm$  95% confidence interval as

normalized to the abundance of GAPDH from all experiments. Scale bars, 5  $\mu\text{m}$ . \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\*\* $P < 0.0001$  by Student's t-test (D to G) or two-way ANOVA (I).



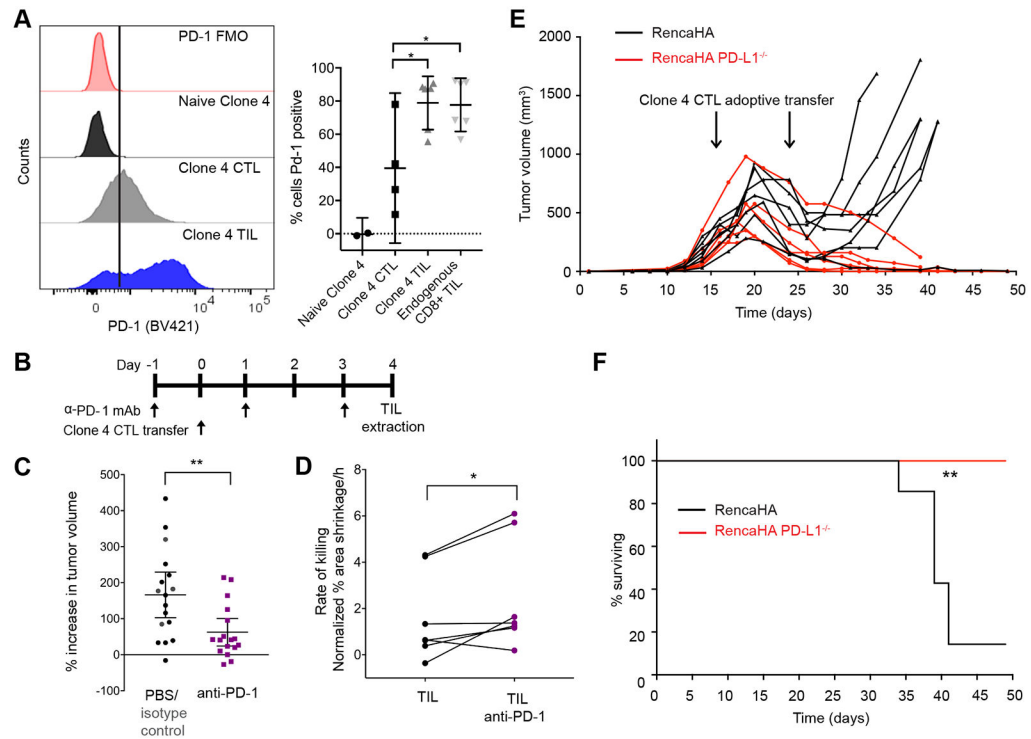
**Fig. 5. Compared to CTLs, TILs display a reduced cytoplasmic Ca<sup>2+</sup> influx.** (A to D) Microscopy analysis of Fura-2–loaded Clone 4 CTLs and TILs interacting with Renca<sup>HA</sup> cells over time. (A and B) DIC (top) and false-colored fluorescence images (bottom) are representative of at least two independent experiments. (C and D) Ratios of Fura-2 emission upon excitation at 340 nm over 380 nm are from five randomly chosen single CTLs and TILs (C) or are means ± SEM from 50 CTLs and 14 TILs from at least two independent experiments (D). (E) Fluorescence microscopy analysis of the killing of Renca<sup>HA</sup> cells in vitro by HA-specific CTLs treated with or without NiCl<sub>2</sub> and BAPTA-AM, as indicated. Average Renca<sup>HA</sup> cell death rate data are means ± SEM from three independent experiments. (F to I) Microscopy analysis of HA-specific CTLs treated with or without NiCl<sub>2</sub> and BAPTA-AM interacting with Renca<sup>HA</sup> cells over time. (F) The cell coupling frequencies are means ± 95% confidence interval of at least three independent experiments. The percentage of Clone 4 CTLs with off-synapse lamellae (G), the time of the first appearance of off-synapse lamellae (H), and the percentage of cells that translocated away from the initial contact site (I) are means ± 95% confidence interval of 138 CTLs (from Fig. 2, F to H) and 31 CTLs after treatment from three independent experiments. (J) Graphic summary of TIL defects. Cell shapes, cytoplasmic Ca<sup>2+</sup> concentrations, and the distributions

of lytic vesicles and the indicated cytoskeletal components and regulators of actin dynamics are given schematically for CTLs (left) and TILs (right) at 1 to 3 min of tight cell coupling. Scale bars, 5  $\mu\text{m}$ . \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$  by Student's t-test (D and F), Mann Whitney u-test (H), or proportion's z-test (G and I).



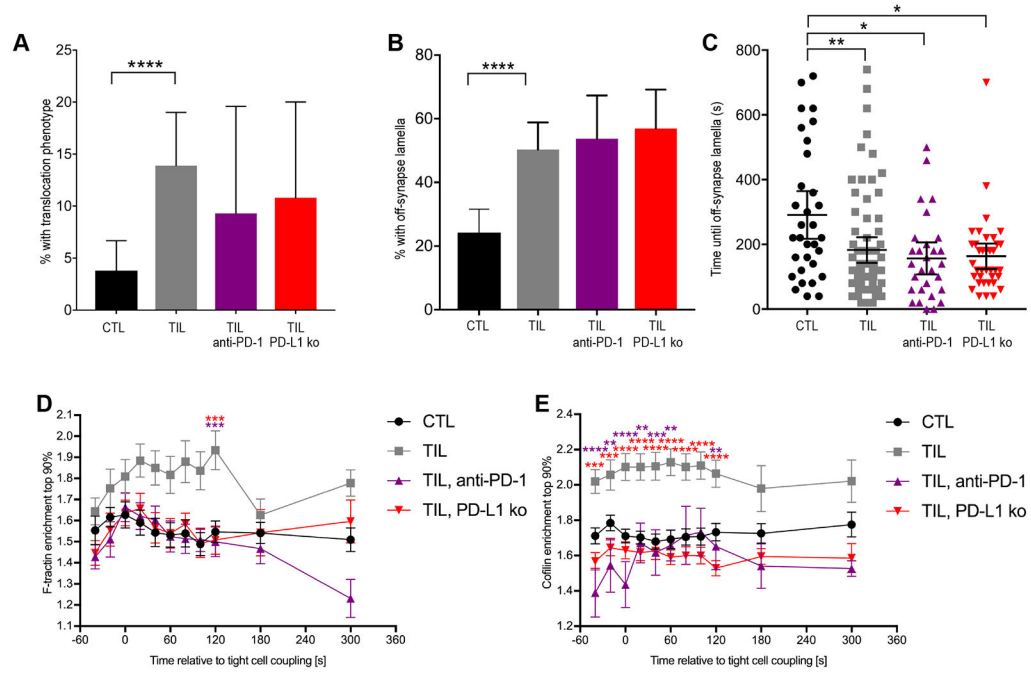
**Fig. 6. Co-culture of CTLs with Renca tumor cell spheroids induces a polarization-impaired state.**

(A) Two-photon microscopy analysis of the infiltration of Clone 4 CTLs into spheroids overnight. The 2D section through the center of a spheroid of mCherry-Renca cells (red) with calcein-labeled, HA-specific SILs (green) is representative of three independent experiments. (B) Fluorescence microscopy analysis of the killing of Renca<sup>HA</sup> cells in vitro by HA-specific CTLs and SILs. Average Renca<sup>HA</sup> cell death rate data are means ± SEM from seven independent experiments. (C) Flow cytometric analysis of the relative expression of PD-1 on HA-specific CTLs and SILs. Data are normalized medians ± 95% confidence interval from three independent experiments. (D to F) Microscopy analysis of HA-specific CTLs and SILs interacting with Renca<sup>HA</sup> cells over time. The percentage of cells with off-synapse lamellae (D), time of the first occurrence of off-synapse lamellae (E), and the percentage of cells that translocated away from the contact site (F) are means ± 95% confidence interval of 138 CTLs (from Fig. 2, F to H) and 43 SILs from three independent experiments. (G) Fluorescence microscopy analysis of Fura-2-loaded HA-specific CTLs and SILs interacting with HA peptide-pulsed Renca<sup>HA</sup> cells over time. Normalized Fura-2 emission data are means ± SEM from 50 CTLs (from Fig. 5D) and 40 SILs from three independent experiments. Scale bar, 100 μm. \**P* < 0.05, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 by paired Student's t-test (B), Student's t-test with Welch's correction (C), proportion's z-test (D and F), Mann Whitney u-test (E), or Student's t-test (G).



**Fig. 7. Loss of PD-1 engagement in vivo improves TIL killing ability.**

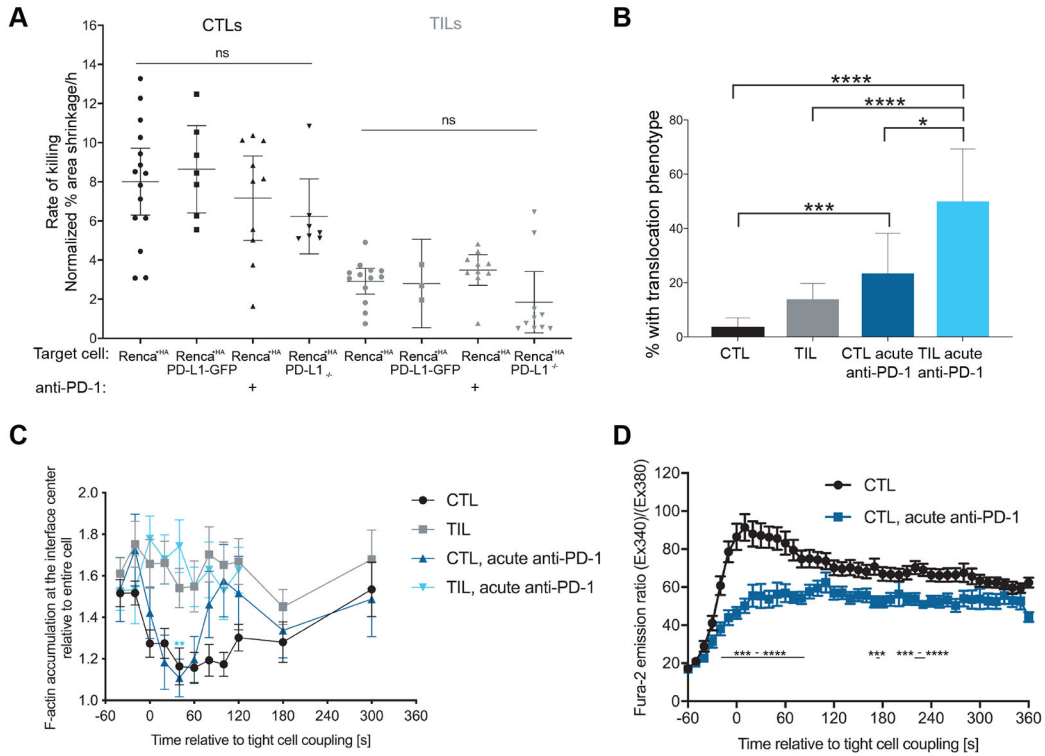
(A) Flow cytometric analysis of the cell surface expression of PD-1 on naïve and primed Clone 4 CTLs, Clone 4 TILs, and endogenous CD8<sup>+</sup> TILs. Left: Histograms are representative of at least two independent experiments. Right: Data are means ± 95% confidence interval. (B and C) Analysis of subcutaneous RencaHA tumor growth in mice after treatment with antibody against PD-1 and adoptive transfer of Clone 4 CTLs, as indicated. The percentage increase in tumor volume within 5 days of the initiation of antibody treatment for 17 mice/condition with means ± 95% confidence interval are from five independent experiments. (D) Fluorescence microscopy analysis of the killing of Renca<sup>+HA</sup> cells in vitro by HA-specific TILs from untreated control mice or mice treated with antibodies against PD-1. Average Renca<sup>+HA</sup> cell death rate data with means ± 95% confidence interval are from six independent experiments. (E and F) Analysis of subcutaneous RencaHA and RencaHA PD-L1<sup>-/-</sup> tumor growth in mice after adoptive transfer of HA-specific CTLs at the indicated times. Tumor volume (E) and survival (F) data of 7 RencaHA and 6 RencaHA PD-L1<sup>-/-</sup> tumor-bearing mice are pooled from two independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  by one-way ANOVA (A), Mann Whitney u-test (C), paired Student’s t-test (D), or Mantel-Cox test (F).



**Fig. 8. Loss of PD-1 engagement in vivo partially rescues cytoskeletal polarization.**

(A) Fluorescence microscopy analysis of F-tractin-GFP-expressing HA-specific CTLs and TILs from the indicated mice interacting with Renca<sup>HA</sup> cells. Data showing the accumulation of F-actin at the center of the immune synapse are means ± SEM of 60 CTLs, 51 TILs (from Fig. 3D), 42 TILs from mice treated with antibody against PD-1, and 43 TILs from RencaHA PD-L1<sup>-/-</sup> tumors from two independent experiments. (B to D) Fluorescence microscopy analysis of cofilin-GFP-expressing HA-specific CTLs and TILs from the indicated mice interacting with HA peptide-pulsed Renca<sup>HA</sup> cells. Data showing the accumulation of cofilin-GFP at the interface at individual time points (B) and at early (C) and late (D) times are means ± SEM (B) or are means ± 95% confidence interval (C and D) of 90 CTLs, 58 TILs (from Fig. 4D), 32 TILs from mice treated with antibody against PD-1, and 55 TILs from RencaHA PD-L1<sup>-/-</sup> tumors from at least two independent experiments. \**P* < 0.05, \*\**P* < 0.01 (D), \*\*\**P* < 0.0045 (A, to account for Bonferroni correction) by one-way ANOVA.





**Fig. 9. Acute in vitro blockade of PD-1 does not restore killing or cytoskeletal polarization.** (A) Fluorescence microscopy analysis of the killing of Renca<sup>HA</sup>, Renca<sup>HA</sup> PD-L1<sup>-/-</sup>, and Renca<sup>HA</sup> PD-L1-GFP cells in vitro by HA-specific CTLs and TILs in the presence or absence of antibody against PD-1, as indicated. Average Renca<sup>HA</sup> cell death rates with means ± 95% confidence interval are from at least four independent experiments. (B) Microscopy analysis of HA-specific CTLs and TILs interacting with HA peptide-pulsed Renca<sup>HA</sup> cells in the presence or absence of antibody against PD-1, as indicated. The percentage of cells that translocated away from the contact site are means ± 95% confidence interval of 138 CTLs, 137 TILs (from Fig. 2, F to H), 35 CTLs treated with anti-PD-1, and 24 TILs treated with anti-PD-1 from two independent experiments. (C) Fluorescence microscopy analysis of F-tractin-GFP-expressing HA-specific CTLs and TILs interacting with HA peptide-pulsed Renca<sup>HA</sup> cells in the presence or absence of antibody against PD-1, as indicated. Data showing the accumulation of F-tractin-GFP data at the center of the immune synapse are means ± SEM of 60 CTLs, 51 TILs (from Fig. 3D), 41 CTLs+anti-PD-1, and 28 C TILs+anti-PD-1 from two independent experiments. (D) Fluorescence microscopy analysis of Fura-2-loaded, HA-specific CTLs with Renca<sup>HA</sup> cells in the presence or absence of antibody against PD-1, as indicated. Normalized Fura-2 emission data are means ± SEM of 50 CTLs (from Fig. 5D) and 52 CTLs treated with anti-PD-1 from two independent experiments. \**P* < 0.05, \*\**P* < 0.0045 (to account for Bonferroni correction), \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001; by Kruskal-Wallis test with comparison to ‘Renca<sup>HA</sup>’ (A, CTL), Kruskal-Wallis test and one-way ANOVA because it is difficult to satisfy all assumptions for either test (A, TIL) proportion’s z-test (B), one-way ANOVA (C), or Student’s t-test (D).