Ultrarapid lytic granule release from CTLs activates Ca\textsuperscript{2+}-dependent synaptic resistance pathways in melanoma cells

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Human cytotoxic T lymphocytes (CTLs) exhibit ultrarapid lytic granule secretion, but whether melanoma cells mobilize defense mechanisms with commensurate rapidity remains unknown. We used single-cell time-lapse microscopy to offer high spatiotemporal resolution analyses of subcellular events in melanoma cells upon CTL attack. Target cell perforation initiated an intracellular Ca\textsuperscript{2+} wave that propagated outward from the synapse within milliseconds and triggered lysosomal mobilization to the synapse, facilitating membrane repair and conferring resistance to CTL induced cytolysis. Inhibition of Ca\textsuperscript{2+} flux and silencing of synaptotagmin VII limited synaptic lysosomal exposure and enhanced cytotoxicity. Multiplexed immunohistochemistry of patient melanoma nodules combined with automated image analysis showed that melanoma cells facing CD8\textsuperscript{+} CTLs in the tumor periphery or peritumoral area exhibited significant lysosomal enrichment. Our results identified synaptic Ca\textsuperscript{2+} entry as the definitive trigger for lysosomal deployment to the synapse upon CTL attack and highlighted an unpredicted defensive topology of lysosome distribution in melanoma nodules.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are a vital component of the immune response against tumor cells, acting via the rapid and highly localized release of lytic molecules (e.g., perforin and granzymes) within specialized secretory regions termed immunological or lytic synapses (1–6). Upon CTL attack, the formation of perforin pores in the target cell membrane facilitates the entry of granzyme B and other serine proteases, which ultimately initiate target cell apoptosis (7, 8).

CTL cytotoxic responses are highly sensitive, rapid, and efficient and can be triggered by the recognition of a very small number of specific peptide major histocompatibility complex (pMHC) complexes on the target cell surface (3, 9–11). Upon engagement of the T cell receptor (TCR) with pMHC, TCR-mediated signaling initiates cytoskeletal remodeling that culminates in the polarization of the microtubule organizing center (MTOC) and lytic granules toward the lytic synapse, enabling the directional release of cytotoxic factors (12–15). In addition, human CTLs also possess a distinct mechanism of ultrarapid lytic granule secretion that is deployed within seconds of TCR engagement; this process is uncoupled from MTOC repolarization and allows CTLs to annihilate multiple targets simultaneously (2, 16).

Despite the high sensitivity, rapidity, and efficacy of lytic granule secretion, CTLs are inefficient at eliminating tumor cells; analyses at the single-cell level both in vitro and in vivo have confirmed that lethal hit delivery is a relatively rare outcome of CTL:tumor cell interaction, whereas tumor cell recovery following sublethal hit delivery is very common (17, 18). Such recovery events are attributable to the diverse resistance mechanisms developed by tumor cells to counteract CTL attack both at the immunological synapse (IS) and more broadly in the context of apoptosis evasion (19–22). Among these specific resistance mechanisms, melanoma cells have been shown to exhibit a synaptic defense mechanism based on highly localized lysosome exocytosis and perforin degradation (23). Because such resistance mechanisms are engaged downstream of successful antigen presentation, IS formation, and lytic granule release, even the most effective immune checkpoint inhibitors are subject to therapeutic failure if these downstream resistance mechanisms are not overcome. Recent reports have also indicated that failed apoptosis increases the aggressiveness of melanoma cells (24), highlighting the therapeutic importance of delivering a decisive lethal blow upon CTL attack. As such, identifying and countering molecular resistance mechanisms at the lytic synapse remains an urgent challenge in the context of tumor immunotherapy.

We hypothesized that the capacity of human CTLs to undergo rapid lytic granule secretion immediately after conjugate formation with target cells hinders the efficient killing of melanoma cells by activating near-instantaneous synaptic resistance pathways. Although this swift initial release of a few lytic granules is useful to kill multiple sensitive target cells encountered simultaneously or in series (16), these early membrane injury events may serve as a danger signal for melanoma cells, enabling them to activate highly effective and targeted resistance pathways capable of countering the full force of CTL attack.
In the present study, we investigated with unprecedented temporal resolution the immediate downstream signaling pathways triggered by membrane perforation that serve to rapidly engage and localize synaptic defense mechanisms in melanoma cells. Our results show that initial lytic granule secretion by human CTLs and the resultant perforin-dependent membrane injury trigger an ultrarapid wave of $[Ca^{2+}]_i$ entry into melanoma cells originating at the lytic synapse. This signaling cascade initiates targeted vesicular trafficking and immediate deployment of localized lysosome-based membrane repair mechanisms to the synapse. We also used clinical cohorts of melanoma patients to assess the relevance of these repair mechanisms in vivo. These analyses revealed that melanoma nodules in vivo adopt an unexpected configuration in which lysosome-dense melanoma cells face the CTL-enriched microenvironment, highlighting the potential relevance of this molecular defense mechanism in the clinical context.

RESULTS

Lethal hit delivery to melanoma cells comprises an initial ultrarapid step

Before analyzing the response of individual melanoma cells to CTL lethal hit delivery, we first sought to quantify the rapidity of CTL lytic granule release when faced with antigen-pulsed D10 melanoma cells, a system in which target cells effectively resist CTL-induced cytotoxicity (23). To accomplish this, we used CD107a exposure on the CTL surface, a well-accepted readout for CTL lytic granule secretion (25). As shown in Fig. 1A, we demonstrated a highly sensitive, rapid, and dose-dependent CTL response to antigen-pulsed D10 melanoma cells at different time points following conjugate formation, with more than 25% of CTLs demonstrating evidence of granule release within 2 min at the highest antigen concentration tested. To validate these observations, we conceived a novel method to detect exocytosis events in CTLs using FM1-43, a well-characterized fluorescent lipophilic dye that intercalates into the phospholipid bilayer upon the induction of membrane turnover (26). This compound was added to culture medium during the conjugation of CTLs with nonpulsed or antigen-pulsed D10 cells. FM1-43 fluorescence was measured in CTLs by flow cytometry at multiple time points following conjugate formation (gating strategy illustrated in fig. S1). Under nonpulsed conditions, a basal uptake of FM1-43 by CTLs could be observed. This uptake was rapidly increased in a substantial proportion of CTLs upon interaction with cognate antigen-presenting melanoma cells, an observation that we validated using four different melanoma cell lines as targets (Fig. 1, B and C). To verify the sensitivity of FM1-43 uptake as a readout of the extent and rapidity of lytic granule secretion, CTLs were next conjugated with D10 melanoma cells pulsed with diminishing concentrations of antigenic peptide. This analysis demonstrated that surface expression of CD107a and uptake of FM1-43 increased in parallel, at each time point tested (Fig. 1, A and D), thus validating FM1-43 uptake as a highly sensitive method to study ultrarapid lytic granule secretion (L. Filali et al. filed patent; PCT/EP2019/082672). Moreover, FM1-43 uptake showed comparable rapidity and sensitivity to CD107a exposure, a highly validated and widely used readout for degranulation (Fig. 1, A and D). At all antigen concentrations tested, FM1-43 uptake within CTL plasma membrane could be detected as early as 2 min after CTL/target cell conjugation, and almost half of CTLs demonstrated FM1-43 uptake within 2 min at the highest antigen concentration tested (Fig. 1D). The above results solidify the notion that initial lytic granule secretion by CTLs is extraordinarily rapid, even when CTLs face cytotoxicity-resistant melanoma cells.

$Ca^{2+}$ waves are triggered on the melanoma cell side of the lytic synapse

While ultrarapid lytic granule secretion is instrumental for killing multiple sensitive target cells encountered simultaneously (2, 16), it might be detrimental when facing cytotoxicity-resistant targets such as melanoma cells, which are capable of mobilizing defensive molecules to the lytic synapse with extreme rapidity (23). We therefore investigated in real time whether this initial lytic granule secretion might initiate a $Ca^{2+}$-mediated signaling cascade in melanoma cells that in turn engages rapid defense/reparation responses. It is known that target cells undergo $[Ca^{2+}]_i$ increase upon CTL attack as a result of perforin-mediated plasma membrane damage (2, 16, 27, 28). Yet, previous time-lapse studies were not performed with sufficiently high temporal resolution to define the rapidity and the spatial characteristics of $[Ca^{2+}]_i$ increase in target cells under CTL attack.

To overcome this limitation, we imaged CTL/melanoma cell conjugates using spinning-disk time-lapse microscopy at a rate of 16 images/s, which allowed us to examine the $[Ca^{2+}]_i$ increase at high spatiotemporal resolution. For these experiments, CTLs were loaded with Tubulin Tracker Green to visualize microtubule dynamics and D10 cells were loaded with Fluo-4 acetoxyethyl ester (AM) to visualize $[Ca^{2+}]_i$. As shown in fig. S2, Fluo-4-AM staining is visible in D10 cells under basal conditions as expected.

Figure 2A and movie S1 illustrate the rapid $[Ca^{2+}]_i$ increase, which occurs in melanoma cells upon CTL attack. This phenomenon was detected before the repolarization of the MTOC (around which the majority of lytic granules are clustered) toward the synapse. Accordingly, as shown in fig. S3, in several additional CTL/target cell conjugates, the MTOC was still several micrometers away from the IS at the time of $[Ca^{2+}]_i$ increase, suggesting that synaptic release of MTOC-associated granules had not yet occurred. These data were in line with our previous reports obtained using nontumor target cells (2). Of interest, the snapshots in Fig. 2A appear to indicate that the $[Ca^{2+}]_i$ increase originates in the synaptic area and subsequently propagates outward through the melanoma cell. This is particularly apparent in the CTL/target cell conjugate shown in Fig. 2B, wherein three discrete hotspots of $Ca^{2+}$ entry into the target cell were clearly apparent immediately following contact with the CTL (Fig. 2B and movie S2).

On the basis of the rapid intensification of the Fluo-4-AM fluorescence signal, the approach used in this study is much faster than ratiometric measurements that we and others have used in the past to measure $[Ca^{2+}]_i$, using time-lapse microscopy (28). Nevertheless, this fluorochrome has the potential limitation that, being a single channel dye, its signal can be sensitive to changes in focal plane or differences in sample thickness. Although this potential artifact is unlikely in adherent cells such as melanoma cells, to exclude this possibility, we nevertheless measured $[Ca^{2+}]_i$ in multiple CTL/target cell conjugates and systematically quantified Fluo-4-AM fluorescence intensity in both the synaptic and distal areas (Fig. 2C). In the representative example shown in Fig. 2A, the $[Ca^{2+}]_i$ increase was observed first in the synaptic area before being detected in the distal one (Fig. 2D). We used this quantification paradigm to assess a substantial number of CTL/target cell conjugates to calculate the time needed to reach half-maximum Fluo-4-AM fluorescence intensity.
in the synaptic versus distal area of each melanoma cell following CTL attack. Figure 2E demonstrates that the increase in Fluo-4-AM fluorescence intensity was consistently delayed by several hundreds of milliseconds in the distal area compared to the synaptic area, implicating the lytic synapse as the origin of the Ca\textsuperscript{2+} wave. These results also highlighted the rapidity of Ca\textsuperscript{2+} wave propagation through the target cell, demonstrating that this early danger signal was typically transmitted from the synapse throughout the cytoplasm in under a second (Fig. 2E). Together, the above results indicate that ultrarapid Ca\textsuperscript{2+} waves were apparent on the target cell side of the synapse upon CTL attack but before repolarization of CTL lytic machinery.

**Localized Ca\textsuperscript{2+} entry triggers swift melanoma cell lysosome exposure at the lytic synapse**

We then explored whether this ultrarapid [Ca\textsuperscript{2+}]\textsubscript{i} increase in the melanoma cell prompted lysosome exposure at the lytic synapse and whether this might occur before CTL repolarization. To address this question, D10 cells were transfected with in vitro transcribed mRNA encoding the transmembrane portion of the lysosomal protein CD107a coupled to mApple/SEpHluorin. This biosensor appears red in acidic vesicles (when the pH-sensitive SEpHluorin probe does not emit fluorescence but mApple appears red) but turns yellow under neutral conditions (when the SEpHluorin probe appears green and mApple appears red, overlap appears yellow); this feature permits the visualization of lysosomal exposure on cell surface associated with an increase in the green fluorescence intensity of SEpHluorin that occurs as the probe encounters a neutral environment (29). To first verify the pH sensitivity of our probe, we confirmed that SEpHluorin green fluorescence intensity was enhanced as expected following treatment with NH\textsubscript{4}Cl (which increases cellular pH) (fig. S4) (30). Subsequent live cell microscopy of transfected melanoma cells revealed a constitutive exposure of CD107a\textsuperscript{+} vesicles on the cell surface before interaction with cognate CTLs, as indicated by modest SEpHluorin signal (yellow) at the cell surface (fig. S5A). This is
consistent with the known enhancement of vesicular trafficking in melanoma compared to nontransformed cells (31). Upon conjugation with CTL, an increased and localized exposure of mApple/SEpHluorin-tagged CD107a was apparent at the synapse (Fig. 3, A to C). This occurred extremely quickly, as demonstrated in the examples shown in Fig. 3A, in Fig. S5 (A and B), and in movies S3 to S5. Examination of multiple CTL/melanoma cell conjugates revealed that this synaptic CD107a⁺ vesicle exposure could be detected predominantly before, and rarely simultaneously with, MTOC repolarization within the CTLs (Fig. 3D).

To expand upon these observations, we examined the functional role of Ca²⁺ signaling in the observed synaptic exposure of mApple/SEpHluorin-tagged CD107a. To accomplish this, we used the well-characterized calcium chelator 1,2-bis(2-aminophenoxy)ethane-

N,N,N’,N’-tetraacetic acid (BAPTA)-AM. As shown in Fig. S6, 50 μM BAPTA-AM was sufficient to abrogate calcium flux in the vast majority of cells tested; a minority of cells exposed to BAPTA-AM were incompletely buffered and still underwent calcium flux, although the time to calcium flux was significantly increased. As illustrated in Fig. 3E and movie S6, pretreating D10 cells with BAPTA-AM...
Fig. 3. Rapid Ca\(^{2+}\)-dependent melanoma cell synaptic lysosomal exposure after CTL attack. D10 cells were first transfected with mRNA coding for CD107a-mApple-SEpHluorin and then conjugated with Tubulin ViaFluor 647–labeled CTLs. In the indicated experiments, caspase-3 substrate was also added to the medium. (A) Snapshots illustrating mApple (red), pHluorin (green, arrows), and tubulin (dark blue, arrows) fluorescence upon D10 cell contact with CTL. (B) Gating strategy for pHluorin fluorescence intensity analysis in the synaptic area. (C) pHluorin fluorescence intensity as a function of time in the synaptic area following contact with cognate CTLs. (D) Time required to detect pHluorin fluorescence in D10 cells versus time required to detect CTL MTOC polarization. pHluorin green fluorescence synaptic signal could be detected in a subset of inspected antigen-specific CTL/melanoma cell conjugates from three independent experiments. (E and F) CD107a-mApple-pHluorin–tagged melanoma cells were pretreated or not with 50 \(\mu\)M 1,2-bis(2-aminophenoxy)ethane-\(N,N,N',N'\)-tetraacetic acid (BAPTA)–AM and conjugated with cognate CTLs. (E) Snapshots depicting a CTL entering in contact with a BAPTA-AM–pretreated peptide-pulsed melanoma cell. (F) Percentage of melanoma cells either untreated or treated with BAPTA-AM showing synaptic pHluorin staining or caspase-3 staining. Fifteen untreated conjugates and 16 BAPTA-AM–pretreated conjugates were analyzed from three independent experiments. Wilcoxon paired \(t\) test was used to assess the statistical significance of differences. **\(P < 0.01\). See also movies S4 and S6.
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reduced the defensive exposure of CD107a+ lysosomes at the lytic synapse and instead triggered the onset of apoptosis, characterized by widespread lysosome permeabilization (indicated by the profound increase in SEPHluorin green fluorescence intensity resulting from the loss of lysosomal acidity) and caspase-3 activation (Fig. 3E) (32, 33). Figure 3F shows the effect of BAPTA-AM on lysosome synaptic exposure and caspase-3 activation in multiple CTL/target cell conjugates. At the single-cell level, our results clearly illustrate that melanoma cell reparative synaptic responses are very rapid and frequently precede CTL repolarization responses. This observation also highlights that the release of MTOC-associated lytic vesicles, which comprise the bulk of lytic vesicles within the CTL, is not required for robust and highly effective melanoma resistance mechanisms to be engaged. These observations also establish a definitive mechanistic link between localized and rapid Ca2+ entry, lysosomal exposure, and melanoma cell resistance to CTL-induced cytotoxicity.

**Perforin-mediated pore formation triggers melanoma cell reparative membrane turnover**

Together, the above results show that, upon CTL-melanoma cell conjugation, CTLs undergo an initial rapid step of lytic granule secretion and, at the same time, melanoma cells respond with Ca2+-dependent lysosome exocytosis. To define whether a mechanistic link existed between these two rapid responses, we investigated melanoma interaction with CTLs in which the perforin pathway was silenced.

To address this question, we profited from the method based on FM1-43 incorporation into the plasma membrane of cells undergoing vesicular trafficking that we validated previously to study early secretory events in CTLs (Fig. 1). Given that Ca2+-dependent lysosome exocytosis is a crucial component of plasma membrane wound healing (34), we reasoned that by measuring the rate of FM1-43 incorporation in target cells as a readout for endocytic/exocytic turnover, we could assess the kinetics of membrane repair in a sensitive, high-throughput manner. We performed this analysis on D10 cell and on three additional melanoma cell lines LB2259, EB81, and M17 by focusing on the target cells in our fluorescence-activated cell sorting (FACS) analysis experiments (fig. S1). In the absence of the antigenic peptide, melanoma cells underwent a basal uptake of FM1-43 that was strongly enhanced in a time-dependent manner in peptide-pulsed melanoma cells conjugated with cognate CTLs (Fig. 4, A and B), indicating rapid melanoma cell membrane reparative turnover. To verify whether FM1-43 uptake was likewise apparent in target cells pulsed with low concentrations of antigenic peptide, we repeated this analysis using a range of peptide concentrations (Fig. 3F) and time points tested relative to the nonpulsed condition (fig. S7).

Using this live-cell imaging setup, we monitored FM4-64 signal intensity over time (movie S7) and observed that, while FM4-64 was taken up at low levels in both the target and CTLs at baseline (Fig. 5A, top left, baseline, t = 1.25 min), upon synapse formation, membrane turnover increased substantially at the interface between the two cells (Fig. 5A, bottom left, IS formation, t = 12.39 min).

Since the repair of injured eukaryotic plasma membranes is known to be Ca2+ dependent (34), we assessed whether interfering with the Ca2+ pathway would affect melanoma cell membrane turnover. D10 cells were again pretreated with the Ca2+ chelator BAPTA-AM (50 μM for 2 hours at 37°C) or solvent control (DMSO, dimethyl sulfoxide) and FM1-43 uptake was quantified by flow cytometry following conjugation with cognate CTL for the indicated times. This analysis revealed that membrane turnover was significantly inhibited by Ca2+ chelation, verifying that FM1-43 incorporation, similarly to lysosome exocytosis (Fig. 3), is Ca2+ dependent (Fig. 4C).

**Evidence of synaptic membrane turnover at the individual cell level**

We next investigated the localization of lipophilic dye uptake at the single-cell level using time-lapse spinning disk microscopy. CTLs were preloaded with PKH-67 (green), a membrane dye used to demarcate the CTL during interaction with D10 cells. FM4-64 (an analog of FM1-43 with a different emission wavelength, shown in pseudocolor) was added into the culture medium at the start of the time-lapse acquisition and maintained throughout the assay. Using this live-cell imaging setup, we monitored FM4-64 signal intensity over time (movie S7) and observed that, while FM4-64 was taken up at low levels in both the target and CTLs at baseline (Fig. 5A, top left, baseline, t = 1.25 min), upon synapse formation, membrane turnover increased substantially at the interface between the two cells (Fig. 5A, bottom left, IS formation, t = 12.39 min).

To assess this phenomenon quantitatively, we first generated a single-pixel line trace of both PKH-67 and FM4-64 signal intensity [representative examples are shown in fig. S9 (A to D)]. However, because a single-pixel line trace is prone to stochastic variability, we expanded the analysis to include the full width of the synapse. To
accomplish this, we manually assigned elliptical masks to CTL and D10 cells at both time points (Fig. 5B), using the PKH-67 signal (shown in green) to identify the CTL. Next, we automatically defined a region of interest (ROI) consisting of a 32 × 16 pixel rectangle that encompassed the area around the interface between the target and the CTL, which was aligned along the axis of the two cells (Fig. 5, A and B, yellow rectangle). We then calculated the average FM4-64 signal intensity at each point across the 32-pixel length of this rectangle (Fig. 5B; colored bars indicate cell boundaries).

Using this analysis, we demonstrated that, while FM4-64 is taken up at low levels in both cell types at baseline (Fig. 5B, top), upon synapse formation, membrane turnover increases substantially at the interface of the two cells (Fig. 5B, bottom). By calculating the area under the curve (AUC; shown shaded in light red) for each time point, it is apparent that total FM4-64 signal intensity is greater upon synapse formation (AUC = 107.50 fluorescent units) compared to baseline (AUC = 72.31 fluorescent units). This suggests that the peak of FM4-64 signal intensity observed at the interface of the two cells upon synapse formation is not just the sum of the combined baseline signal in each cell.

A second analysis of this phenomenon wherein a single CTL interacts with two target cells is shown in Fig. 5 (C and D). As shown in Fig. 5C and movie S8, when a CTL formed simultaneous contacts with two different melanoma cells, extensive membrane turnover was observed at both separate contacts sites. Figure 5D shows the quantification of FM4-64 signal intensity for each of the two melanoma cells and highlights how the strongest FM4-64 signal was localized to the interface between the two cells upon synapse formation. Again, there is a substantial increase in total FM4-64 signal in the ROI upon synapse formation as measured by the AUC.
Fig. 5. Synaptic membrane turnover in melanoma cells after CTL attack. D10 cells pulsed with antigenic peptide were conjugated in the presence of FM4-64 with CTLs previously stained with PKH-67. (A) Snapshots depicting a melanoma cell interacting with a CTL at baseline (top) and upon IS formation (bottom), with FM4-64 in pseudocolor (left) and FM4-64 in red plus PKH-67 in green (right). (B) Elliptical masks attributed to each target (blue) and CTL (green) for image analysis are depicted. Yellow rectangles indicate region of interest. Average FM4-64 signal intensity across the width of the synapse at each point is shown (dark red line; area under the curve shaded in light red). Colored bars indicate the boundary of the CTL (green) and target (blue), respectively. (C) Snapshots depicting a CTL at baseline (top) and after synapse formation with two D10 cells (bottom). (D) Average FM4-64 signal intensity shown as in (B). (E) Automated image analysis in which FM4-64 was quantified in synaptic and distal areas of each cell. Pixels in which CTL and target cell masks overlapped were excluded from analysis (gray), leaving only the area that could be unambiguously attributed to either cell (thatched). The mean of activated pixels was calculated. Twelve synapses were analyzed using paired t tests. *P < 0.05; ***P < 0.001. ns, not significant. See also movies S7 and S8.
for both melanoma cell #1 (97.24 fluorescence units upon synapse formation versus 73.72 fluorescence units as baseline) and melanoma cell #2 (140.85 fluorescence units upon synapse formation versus 82.83 fluorescence units at baseline). This example provides compelling evidence to suggest that synapse repair mechanisms are preserved even when targets outnumber CTLs, as substantial FM4-64 turnover is observed in both melanoma cells simultaneously.

Collectively, the above results illustrate at the single-cell level that active membrane turnover is initiated at the CTL/melanoma lytic synapse. Nevertheless, most of the signal is obtained where the two cell masks overlap, and because of this, the FM4-64 signal cannot be unambiguously attributed to either cell.

To address this issue, we performed an additional analysis in which only the nonoverlapping areas were quantified and areas where the cell masks overlapped were excluded from analysis. As illustrated in the schematic in Fig. 5E, the target (blue) and CTL (green) were each manually assigned an elliptical mask at two distinct time points (at first contact and at synapse formation) and four rectangular regions of interest (ROI) were defined for both time points: (i) target cell synapse, (ii) target cell distal, (iii) CTL synapse, and (iv) CTL distal, each 30 × 8 pixels. Within the synaptic ROIs, the pixels in which the CTL and target cell masks overlapped were excluded from analysis (gray), leaving only the area that could be unambiguously attributed to either the CTL or the target (indicated by the thatched area in schematic). As shown in Fig. 5E, the MFI significantly increased in the synaptic area of both CTL and target cell upon IS formation compared to first contact, indicating localized membrane turnover on both sides of the IS. This increase in localized membrane turnover is still observed although most of the FM4-64 signal (located where the two cells overlap) is eliminated from this follow-up analysis. In the distal areas, no significant change in FM4-64 fluorescence was observed. These results recapitulate the flow cytometry data shown in Figs. 1 and 4 and fig. S7, which collectively demonstrate that membrane turnover was observed in both CTLs and target cells during conjugation and provide further validation that this membrane turnover is localized to the synaptic area of both CTL and target.

**Ca^{2+} pathway directs melanoma resistance at whole population level**

We had initially hypothesized that the capacity of CTLs to undergo rapid lytic granule secretion immediately after conjugate formation with target cells hinders the efficient killing of melanoma cells by activating highly effective, localized synaptic resistance pathways. Since we have shown these localized synaptic resistance pathways to be Ca^{2+}-dependent, we further hypothesized that inhibition of Ca^{2+} signaling either pharmacologically or genetically could disable melanoma synaptic resistance mechanisms and render the cells incapable of withstanding CTL attack. To investigate this hypothesis, we pretreated D10 cells with 50 μM BAPTA-AM (2 hours at 37°C) before conjugation with CTLs and measured CTL-mediated cytotoxicity by flow cytometry. As shown in Fig. 6A, inhibition of Ca^{2+} signaling with BAPTA-AM resulted in increased sensitivity of D10 cells to CTL-mediated cytotoxicity. This observation was confirmed in three additional melanoma cell lines (Fig. 6B). No significant differences were observed between nonpulsed BAPTA-AM–treated and nonpulsed solvent-treated cells [analysis of variance (ANOVA) with Tukey’s test for multiple comparisons], and 50 μM BAPTA-AM alone (in the absence of CTLs) was shown to be nontoxic to D10 cells, in either the presence or absence of antigenic peptide (fig. S10).

To assess this phenomenon using a genetic approach, we also silenced the expression of synaptotagmin VII (SYTVII), a Ca^{2+} sensor implicated in lysosome exocytosis (38, 39), using a short hairpin RNA (shRNA) approach. D10 cells transfected with SYTVII-specific shRNA showed an 80–90% reduction of SYTVII expression (compared to non-targeting shRNA-transfected control cells) as indicated by real-time quantitative polymerase chain reaction (RT-qPCR) and immunoblot (fig. S11, A and B). As expected, SYTVII-silenced melanoma cells demonstrated enhanced sensitivity to CTL-mediated killing compared to nontargeting shRNA–transfected control cells (Fig. 6C), reinforcing that interruption of intracellular Ca^{2+} sensing pathways is sufficient to significantly impair melanoma resistance mechanisms.

To interrogate this observation in more detail, we assessed whether reparative membrane turnover was dependent on intact Ca^{2+} signaling in the target cell, and thus whether reparative membrane turnover...

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**Fig. 6. Ca^{2+} pathway inhibition in melanoma cells increases their susceptibility to CTL attack.** (A and B) Melanoma cells were either left untreated or pretreated with 50 μM BAPTA-AM for 2 hours in the presence or absence of antigenic peptide and subsequently conjugated for 4 hours with cognate CTLs. Cytotoxicity was assessed using 7-aminoactinomycin D (7-AAD) uptake in target cells by flow cytometry. Sensitive JY cells served as a control for CTL cytotoxicity. Data shown are % of dead (7-AAD+) cells following the 4-hour conjugation. (A) D10 cells or JY cells; (B) EB81, M17, or LB2259 cells. (C) Transfected D10 cells with short hairpin RNA control or silencing SYTVII were either left untreated or pretreated with 50 μM BAPTA-AM (2 hours at 37°C) before conjugation with CTLs and measured CTL-mediated cytotoxicity by flow cytometry. Data shown are % of dead (7-AAD+) cells following the 4-hour conjugation. Data from three independent experiments are expressed as means ± SEM. Wilcoxon test (A and B) or Mann-Whitney test (C) was used to assess statistical significance of differences between groups. *P < 0.05; **P < 0.01.
was responsible for the observed increase in susceptibility of SYTVII-silenced cells to CTL attack. SYTVII-silenced D10 cells demonstrated a significant decrease in FM1-43 incorporation following conjugation with CTLs (fig. S12), which strongly implicated decreased reenerative membrane turnover as the mechanism for the observed increase in cytotoxicity upon silencing of SYTVII (Fig. 6C).

### High CD107a expression at the periphery of melanoma nodules

Our results highlighted the capacity of melanoma cells to respond to CTL attack by ultrarapid Ca\textsuperscript{2+}-dependent membrane turnover and lysosome exocytosis. They were obtained on melanoma cells interacting with antigen-specific human T cell clones using readouts ranging from seconds to hours. We therefore asked whether these findings might be relevant in vivo, under conditions in which human melanoma/immune cell interactions evolve over a period of months to years.

To address this question, we applied multiplexed immunohistochemistry (IHC) to two formalin-fixed, paraffin-embedded (FFPE) tissue sample cohorts of metastatic melanoma patients (table S1). For each sample, the entire tumor fragment and surrounding microenvironment were digitized using a Panoramic 250 Flash II digital microscope (3DHISTECH). All cells were counterstained with hematoxylin. Melanoma cells and T cells were identified by Sox10 (a nuclear marker of melanocytic lineage, orange) and CD8 (purple) expression respectively and cell lysosomal content was identified by CD107a staining (black) (Fig. 7). To prevent the analysis of non-specific artifacts, only Sox10\textsuperscript{high} cells were considered unambiguously to be melanoma cells and included in the analysis.

As shown in typical examples presented in Fig. 7A, both Sox10\textsuperscript{high} melanoma cells (Fig. 7A, top left) and CD8\textsuperscript{+} T cells (Fig. 7A, bottom left) expressed CD107a. This is clearly evident in images taken at the tumor nodule border where these cell populations largely enter in contact (Fig. 7A, top right). To distinguish Sox10\textsuperscript{high} melanoma cells from CD8\textsuperscript{+} T cells and to quantify CD107a expression in individual Sox10\textsuperscript{high} melanoma cells, we developed an image analysis script using Cartesian genetic programming (CGP) adapted for instance segmentation (see Material and Methods). This approach allowed us to quantify, in an automated and nonsubjective fashion, CD107a staining intensity within Sox10\textsuperscript{high} tumor cells along with CD8\textsuperscript{+} T cell infiltration within tumor nodules in the whole-slide image (WSI) at the maximum resolution. To do so, each WSI was cut into a grid of thousands of patches (corresponding to squares of 60 μm, 256 × 256 pixels; Fig. 7A, bottom right, blue square). After color segmentation, based on (HSV) color scale (Fig. 7B, top), instance segmentation (see Material and Methods) allowed us to automatically demarcate individual cells, classify them as either CD8\textsuperscript{+} T cells or Sox10\textsuperscript{high} melanoma cells, and assess lysosomal content at the single-cell level, using the number of positive pixels per cell as a readout (Fig. 7B, bottom, pink). It is important to note that, for the purpose of this analysis, only Sox10\textsuperscript{high} cells were classified as melanoma cells, so as to eliminate Sox10\textsuperscript{low} nonmelanoma cells and nonspecific background staining from the analysis. This strategy was crucial to avoid contaminating our dataset with irrelevant or artifactual data points. In this manner, our analysis was designed to optimize specificity, rather than sensitivity.

The tumor nodules were next split into uniform concentric slices (see Materials and Methods) to define whether CD107a expression varied from the center toward the periphery of the tumor nodule (Fig. 7, C and D). We defined five intratumoral slices (ITS-1–5) per nodule and compared these concentric rings with the tumor center (intratumoral region, ITR). We also included in the analysis the area immediately surrounding the tumor nodule since we observed that individual Sox10\textsuperscript{high} melanoma cells (ranging from a few tens to a few thousands in different patients) could be detected outside the nodule invading the peritumoral area (peritumoral slice, PTS; Fig. 7D). As expected, the PTS was highly enriched in CD8\textsuperscript{+} cells, generating an average effector:target (E:T) ratio of 52.5, whereas the ITR contained only sparse infiltrating CD8\textsuperscript{+} T cells, leading to an average E:T ratio of 0.71 (Fig. 7E). Within the ITS-1, which represented the frontier battleground between the tumor and the microenvironment, the E:T ratio averaged 1.67 (Fig. 7E).

This spatial analysis also enabled us to examine the unique molecular features of the Sox10\textsuperscript{high} melanoma cells located in this frontier battleground, which we hypothesized would bear the molecular signature of prolonged interaction with the adaptive immune system. While CD8\textsuperscript{+} T cells were, as expected, more enriched in the peritumoral area and in the tumor nodule periphery as compared to tumor nodule center (Fig. 7, E and F), Sox10\textsuperscript{high} melanoma cell number as expected was highest in the ITR, with only a few invading cells in the PTS (Fig. 7H). CD107a expression by Sox10\textsuperscript{high} melanoma cells exhibited a statistically significant expression gradient that increased from the tumor center toward the periphery (Fig. 7, G and I). Figure S13 provides an illustrative example of a metastatic melanoma nodule located in a lymph node (fig. S13, A and B) with the cross-sectional expression gradient of CD107a quantified across the whole tumor nodule (fig. S13C). This representative example clearly illustrates that the edges of tumor nodules are populated by Sox10\textsuperscript{high} melanoma cells that are rich in defensive lysosomes, while Sox10\textsuperscript{low} melanoma cells within the ITR had relatively low lysosomal density. As shown in Fig. 7 (G and I), this observation was robustly validated at the level of the whole cohort. It is interesting to note that Sox10\textsuperscript{high} melanoma cells detected outside the nodule invading the PTS (where they were typically surrounded by CD8\textsuperscript{+} T cells) also expressed high CD107a levels indicative of high lysosomal density (Fig. 7, G and I). To determine the proportion of patients that showed a gradient in CD107a staining from the ITR to the PTS-1, the difference in CD107a staining intensity between the two regions was calculated for each patient sample (\(\Delta CD107a = CD107a_{\text{ITS-1}} - CD107a_{\text{ITR}}\)), wherein a positive \(\Delta CD107a\) value indicated an increase in CD107a expression from the interior to the periphery of the tumor. As shown in fig. S14A, the median \(\Delta CD107a\) value for this population of patient samples was greater than 0 (\(P < 0.0001\)), indicating that the CD107a gradient from interior to periphery was observed in the majority of patient samples. The magnitude of this difference (corresponding to the “steepness” of the gradient) was quite variable, highlighting the heterogeneity across different tumor samples. Nonetheless, visualization of these data by quartile (fig. S14B) revealed that >75% of patient samples had a \(\Delta CD107a\) value greater than 0, indicating that, while the absolute difference between the interior and exterior CD107a expression may be heterogeneous, the presence of the gradient was observed in the vast majority of patient samples. Together, these results show that cellular lysosomal content increases from tumor center toward periphery in melanoma nodules, where tumor cells have to face the strongest CD8\textsuperscript{+} T cell attack.
Fig. 7. Increased CD107a staining in Sox10high melanoma cells at the periphery of tumor nodules. (A) Representative melanoma section subjected to multiplexed immunohistochemistry for hematoxylin (blue), Sox10 (yellow), CD8 (purple), and CD107a (black). Top left: Melanoma nodule demonstrating Sox10high melanoma cells with CD107a staining. Top right: Representative image of melanoma nodule border. Invasive melanoma cells are visible beyond the tumor nodule in the peritumoral area. Bottom left: CD8 cell–rich peritumoral area surrounding the tumor nodule. Bottom right: 256 × 256 pixel patch. (B) Overview of CD107a quantitation: Color-based segmentation was performed (top) and the area surrounding melanoma nuclei was demarcated (bottom left), corresponding to the whole melanoma cell and excluding nonmelanoma cells (cyan), after which the number of CD107a+ pixels (pink) in the melanoma intracellular area was counted (bottom right). HSV. (C and D) Decomposition of tumor nodule into concentric slices. ITR, intratumoral region; ITS-1–5, intratumoral slice; PTS, peritumoral slice. (E to H) Quantification of tumor features. Each data point represents the average for an individual tissue sample (n = 90); data shown are means ± SEM. (E) Effector:target ratio between CD8 cells and Sox10high melanoma cells. (F) CD8 cell density. (G) Number of CD107a+ pixels per Sox10high melanoma cell (units = CD107a pixels per cell). (H) Sox10high melanoma cell density. (I) Number of CD107a+ pixels per Sox10high melanoma cell from interior (ITR) to exterior (PTS) (n = 90 tissue samples). A significant linear left-to-right gradient is observed (one-way ANOVA with follow-up test for trend to assess linear relationship across column values). (J) Tabular summary of data analyzed. *P < 0.05; **P < 0.001; ***P < 0.0001.
To determine whether this enrichment in membrane defense mechanisms was unique to melanoma cells or shared with non-transformed cells of the melanocytic lineage, we identified regions of healthy epidermis in our patient slides and examined whether healthy melanocytes in the basal layer of epidermis were likewise enriched in CD107a content. As shown in fig. S15, nontumor-associated melanocytes in the basal layer display robust CD107a immunoreactivity. To determine the functional significance of this observation, we used a nontransformed human melanocyte line (HEMn-MP) in vitro that displayed comparable density of lysosomal proteins CD107a and CD63 to D10 cells (fig. S16A). HEMn-MP cells were then transfected with an HLA-A2-GFP (human leukocyte antigen A2–green fluorescent protein) construct and HLA-A2 expression and plasma membrane localization validated by cytometry and microscopy (fig. S16B). To determine the sensitivity of these cells to CTL attack, peptide-pulsed HEMn-MP cells were conjugated with CTLs and cell death was assessed. Despite fully activating CTLs during antigen presentation as indicated by CD107a surface expression (fig. S16C), the HEMn-MP melanocytes were highly resistant to CTL attack (fig. S16D). These results indicated that lysosomal defense mechanisms against CTL attack might be preserved within the melanocytic lineage, though further study is required to determine whether this feature is enhanced during the processes of tumorigenesis and tumor immunoediting.

DISCUSSION
The rapidity of secretory responses is a hallmark of lethal hit delivery by CTLs. This phenomenon is ordinarily regarded as an advantageous feature of CTL biology, contributing to the rapid and efficient elimination of virally infected or tumorigenic target cells. However, whether the rapidity of lytic granule secretion is beneficial in the context of cancer, or on the contrary, simply induces a commensurately rapid defense response from cytotoxicity-resistant target cells, has not been thoroughly investigated.

In the present study, we offer a high–spatiotemporal resolution analysis of the subcellular events occurring in cytotoxicity-resistant melanoma cells immediately following CTL attack. We demonstrated that CTL attack initiated an ultrarapid intracellular Ca\(^{2+}\) wave that propagated outward from the synapse within milliseconds, in turn triggered defensive lysosome secretion and membrane repair at the synapse. Our results also reveal an unexpected defensive configuration of melanoma nodules in vivo, in which melanoma cells facing CTLs in the tumor periphery or peritumoral area exhibited significant lysosomal enrichment, highlighting the potential in vivo relevance of our findings.

Ca\(^{2+}\) signaling serves a crucial regulatory function in cell biology and is fundamentally essential to the function of immune cells (40). TCR-mediated Ca\(^{2+}\) signaling mediates key cellular processes, such as cell-cell adhesion (41), regulation of signaling enzymes (42), actin cytoskeleton reorganization (43), translocation of transcription factors (44), and lytic granule secretion (45). In CTLs, the kinetics of Ca\(^{2+}\) signaling are influenced by the stability of CTL contact with antigen presenting cells, which in turn finely regulates effector functions. It is well known that either prolonged or sequential IS formation combined with sustained intracellular Ca\(^{2+}\) signaling is required in antigen-stimulated T cells to initiate the nuclear translocation of nuclear factor of activated T-cells (N-FAT), which in turn enhances cytokine gene expression (46–48).

Although the time parameter is well inscribed in the CTL activation paradigm, the spatiotemporal features of Ca\(^{2+}\) signaling in the target cell following CTL attack have yet to be fully defined. Here, we offer initial insights into the nuanced role of Ca\(^{2+}\) signaling in shaping the target cell response to CTL attack. We described the notable capacity of melanoma cells to undertake swift and highly localized reparative membrane turnover and lysosome exocytosis at the IS upon Ca\(^{2+}\) entry, which fortifies the cell against further attack. Ultrarapid live imaging at the single-cell level (Fig. 3) demonstrated that melanoma cells expose their lysosomes at the synapse immediately upon CTL attack, thus mounting a robust and highly localized defense to counter the subsequent arrival of MTOC-associated cytotoxic molecules (2, 16). This observation suggests that the rapidity of the CTL’s initial secretory response may undermine its efficacy when faced with a resistant cell, allowing the target sufficient time to mobilize its defensive resources before the delivery of MTOC-associated cytotoxic molecules. We demonstrated that such Ca\(^{2+}\)-mediated defensive responses play a definitive functional role in melanoma cell resistance, as indicated by the observed increase in sensitivity to CTL-mediated cytotoxicity upon either genetic (shRNA-mediated knockdown of the calcium sensor SYTVII) or pharmacological (BAPTA-AM–mediated calcium chelation) interference with Ca\(^{2+}\) signaling pathways. It is worth noting that, while BAPTA-AM–mediated blockade of the Ca\(^{2+}\) pathway did not entirely abrogate Ca\(^{2+}\) signaling at the indicated doses, it was shown to block calcium flux in the vast majority of target cells tested and significantly delayed (by minutes) calcium flux in the remaining target cells (fig. S6). Complete abrogation of Ca\(^{2+}\) signaling at much higher doses of BAPTA-AM may further increase target cell sensitivity to CTL attack but was not pursued in this context due to potential off-target effects on either CTLs or melanoma cells.

It is intriguing that ultrarapid Ca\(^{2+}\)-mediated responses have been identified on both sides of the IS. It has long been recognized that an intact Ca\(^{2+}\) pathway is functionally necessary for lytic granule release, as illustrated by the impaired CTL lethal hit delivery observed in patients lacking Orai-STIM Ca\(^{2+}\) channels (49). Here, we demonstrated that Ca\(^{2+}\) signaling also mediates a virtually simultaneous and opposing response on the tumor cell side of the lytic synapse, highlighting the relevance of the first moments of CTL/target cell interaction in shaping tumor cell resistance to cytotoxic attack.

It is worth noting that the visualization of Ca\(^{2+}\) signaling through the use of calcium dyes (as we have done with Fluo-4 AM) can itself affect the magnitude or speed of the Ca\(^{2+}\) signaling response, due to potential buffering effects of Fluo-4 AM. This is particularly salient in the context of melanoma cells, which require relatively high concentrations of Fluo-4 AM to avoid active or passive diffusion of the dye out the cells (50). As such, we must consider the possibility that our results represent an understimation of the speed and/or magnitude of the target cell Ca\(^{2+}\) signaling response to CTL attack; nonetheless, it is unlikely that our main observation (that the calcium signal originates at the synapse upon membrane perforation and subsequently spreads throughout the cell) would be affected.

We also demonstrated in our study that membrane turnover as measured by FM1-43 or FM4-64 occurs on both sides of the synapse. FM dyes have been widely used as reagents for the real-time measurement of exocytosis and endocytosis in living neurons and in many kinds of cells (26, 51–53). In our system, FM dye uptake likely reflects membrane turnover and not membrane permeabilization or perforation for several reasons: (i) a steady-state
uptake of FM1-43 occurs in melanoma cells in the absence of the antigenic peptide (a condition in which no permeabilization occurs), and this observation is consistent with heightened baseline vesicular trafficking previously observed in melanoma (31); (ii) the FM dye uptake process is active and Ca2+-dependent, being impaired either by knockdown of SYT7VII in the target cell (fig. S12) or by BAPTA-AM-mediated calcium chelation in target cells (fig. 4), observations that are compatible with an endo/exocytosis process, but not with the passive entry of the compound into the cell upon membrane permeabilization; and (iii) once taken up, FM dye does not rapidly diffuse throughout the cell cytosol, but rather progressively accumulates in a localized manner in the synaptic area and distinct intracellular compartments, which is consistent with vesicular staining. Nonetheless, we acknowledge the limitation that further study is required to fully deconvolve these two possible mechanisms.

Perforin-dependent Ca2+-entry has been reported to trigger in target cells an early endosomal compartment-based repair that favors the induction of apoptosis as opposed to necrosis (8, 27). Our results extend previous observations by demonstrating that Ca2+-entry can also favor cell survival, rather than apoptosis. This finding is compatible with a model in which early perforin binding followed by localized Ca2+-entry and synaptic lysosome exposure protect melanoma cells from CTL-mediated cytotoxicity, even before CTL polarization responses are accomplished.

One of the most compelling results from the current study concerns the observation that intracellular expression of the lysosomal marker CD107a in Sox10high melanoma cells progressively increases from the center to periphery of melanoma tumor nodules. These results were obtained from tissue samples belonging to two independent patient cohorts and were generated using unbiased image analysis with independent evaluation from a clinical pathologist. It is worth highlighting that the CD8+ cell density and distribution identified by automated image analysis in this study corresponds with a remarkable degree of accuracy to that published in other reports (54). For instance, in a representative melanoma nodule assessed using a digital image analysis algorithm developed independently on a 158-patient Italian training cohort, the density of CD8+ cells in the tumor periphery was reported as ~900 CD8+ cells/mm2, decreasing in a stepwise manner toward the tumor core (54); these results closely recapitulate the findings of our study, wherein the T cell density in the tumor periphery was on average 855 CD8+ cells/mm2, decreasing to 455 CD8+ cells/mm2 in the tumor core. These important similarities highlight the external validity of our digital image analysis approach.

Nonetheless, it is worth noting the limitations of the current image analysis pipeline. For example, owing to the stringency of our analysis, which only considered Sox10high melanoma cells, a subset of Sox10low cells of melanocytic origin would have been excluded from our analysis. This strategy was designed to prevent the contamination of the dataset with background artifacts, which automated image analysis cannot yet reliably distinguish from Sox10low cells. Improving the ability of the algorithm to distinguish between nonspecific background staining and Sox10low cells of melanocytic origin remains an area of ongoing research and development. Despite these caveats, there are clear benefits to the automated image analysis strategy used in this study. First, the whole-slide analysis of melanoma nodules from two different cohorts at the maximum resolution generated a rich and robust dataset that permitted the simultaneous analysis of almost 10,000 mm2 of tissue, containing 5.9 × 10^6 individual CD8+ cells and 10.9 × 10^6 Sox10high melanoma cells (fig. 7). The ability to discriminate such a large number of individual cells and classify them according to cell marker expression enabled us to elucidate subtle cell type-specific trends in the expression of protective proteins (in this case, CD107a) that may not have been apparent using more traditional approaches. Moreover, the ability to automatically generate and analyze concentric slices brought to light trends in the spatial distribution of cell defense proteins that may otherwise have remained difficult to discern. Using this approach, we demonstrated strong CD107a expression in Sox10high melanoma cells facing CD8+ T cell infiltrate but not in the ITR where infiltrating CTLs were sparse (fig. 7).

Nonetheless, it is not possible to deduce from our histological data whether this unique distribution of lysosomal content within the tumor landscape represents (i) a redistribution of migratory melanoma cells, with the more lysosome-rich melanoma cells trafficking to the tumor periphery; (ii) a transient up-regulation of lysosomal proteins in CTL-facing melanocytes in the tumor periphery; or (iii) a signature of selective pressure wherein lysosome-rich melanoma cells are selected for and confined to the tumor periphery over a period of months/years. Longitudinal data in which histological samples were available from the same tumor over time would help shed light on this question but are not possible to acquire in the current clinical context.

Similarly, whether lysosome-mediated defense mechanisms that restrict CTL toxicity are “acquired” during tumorigenesis or whether this feature is preserved throughout the melanocyte lineage remains an open question. It is worth noting, however, that melanocytes that were engineered to express HLA-A2 in this study mimicked melanoma cells in their resistance to CTL cytotoxicity (fig. S16), suggesting that this resistance to CTL killing might be lineage-specific, rather than acquired in the context of tumorigenesis. Given the secretory function of healthy melanocytes and the intracellular abundance of lysosome family organelles involved in the synthesis and secretion of melanin, it stands to reason that lysosome-mediated reparative membrane turnover would be a first line of defense against membrane perforation. However, these results do not exclude the acquisition or strengthening of this property over tumoral transformation, or over stages of the disease and immune selection. Further experiments that focus upon the evolution of primary melanocytes and melanomas under the selective pressure of serial CTL exposures would be instrumental to address these questions. Nonetheless, it is apparent that melanocytes in healthy tissues are relatively enriched in lysosomes despite some heterogeneity in their lysosomal content (fig. S15), which further argues for the preservation of lysosomal-mediated defenses throughout the melanocytic lineage.

Our analysis also revealed the existence of a substantial number of Sox10high tumor cells that escaped the tumor nodule and also exhibit strong CD107a expression. The clinical relevance of these cells is presently elusive; however, our current and previous research suggests that high lysosomal content may confer enhanced membrane repair mechanisms that help these cells to resist CTL attack and further investigation into this hypothesis may be warranted.

It is also worth considering that our findings may have implications for immune checkpoint blockade therapy, since melanoma-specific CTLs attacking a tumor nodule are likely to encounter lysosome-rich melanoma cells in the tumor periphery. Although it is premature to speculate on the presence or nature of an association
between lysosomal content and immunotherapy outcome based on our current data, it is interesting to consider that a large-scale, appropriately powered study might be useful to assess whether lysosomal content or distribution might affect the success of immunotherapy. Of course, we acknowledge that the field of biomarker research as it pertains to checkpoint inhibitor response has largely shifted away from attempting to assign predictive value to individual molecules and is instead focusing upon multifactorial predictive markers (55). As an image-rich cohort using multiplex immunohistochemistry, our cohort enables us to visualize the cell type–specific expression and spatial distribution of a single molecule (CD107a) over a large dataset with unprecedented resolution; however, the cohort was not constructed specifically for biomarker research and thus lacks robust annotation of other variables of interest that would be required to perform proper multifactorial predictive marker analyses. For this reason, our dataset is more aptly suited toward hypothesis generation than the prediction of association between individual markers and clinical outcome; nonetheless, we hope that our findings spur further investigation and perhaps provide a rationale for considering lysosomal markers in the design of future studies investigating predictors of response to immunotherapy.

It also remains to be determined whether such defense mechanisms are shared by diverse tumor types. This notion is certainly possible since Ca$^{2+}$-dependent reparative membrane turnover is highly evolutionarily conserved (34). Other cell types might therefore be expected to engage a comparable mechanism to defend themselves against CTL attack. Whether the reparative mechanisms described here prove to be important determinants of outcome during CTL/target cell interaction might depend on the lysosome-specific parameters (e.g., lysosomal mass and basal rate of membrane turnover) as well as the Ca$^{2+}$ signaling–specific parameters (e.g., aberrant expression and function of Ca$^{2+}$ pumps, channels, and binding proteins) of the individual tumor. Both lysosomal homeostasis (56) and calcium signaling networks (57) are known to be perturbed in cancer compared to nontransformed cells, and the specific nature and extent of these perturbations will likely affect the efficacy and kinetics of the Ca$^{2+}$-dependent, lysosome-mediated membrane repair mechanisms engaged during CTL attack. Together, the results of the current study indicate (i) that melanoma cells possess a mechanism allowing them to sense the direction of immune cell attack and to deploy highly localized repair mechanisms to resist further CTL synaptic attacks following the initial release of a few lytic granules, and (ii) that an unexpected topology of high CD107a expression at the periphery of tumor nodules can be found in melanoma clinical samples, suggesting that melanoma cells possess directional strategies to counteract immune cell synaptic attack in vivo. As a consequence, the delivery of soluble packaged lytic molecules, such as cytotoxic molecule–containing exosomes or the recently described supra-molecular attack particles (58, 59), might be a successful strategy to circumvent the synaptic resistance mechanisms described here.

In conclusion, this investigation has highlighted that both the rapidity and spatial localization of Ca$^{2+}$ entry at the IS make it an ideal alert signal to engage ultrarapid defense responses in melanoma cells upon CTL attack. Such insights serve to redefine our understanding of the role of Ca$^{2+}$ signaling in the CTL/target cell interaction paradigm and encourage the contemplation of cell-free cytolytic attack strategies as attractive alternatives to current immunotherapeutic approaches.

**MATERIALS AND METHODS**

**Cell culture**

Cell culture was performed as previously described (60). Human CD8$^+$ T cell lines were purified from healthy donor blood samples using the RosetteSep Human CD8$^+$ T Cell Enrichment Cocktail (StemCell Technologies). For cloning, HLA-A2–restricted CD8$^+$ T cells specific for the NLVPVMVATV peptide or the VLAELVKQI peptide of the cytomegalovirus protein pp65 were single cell–sorted into 96-U-bottom plates using a BD FACSaria II cell sorter using tetramer staining. Cells were cultured in RPMI 1640 medium supplemented with 5% human AB serum (Institut de Biotechnologies Jacques Boy), 50 µM 2-mercaptoethanol, 10 mM Hepes, 1× MEM-Nonspecific Amino Acids (MEM-NEAA) Solution (Gibco), 1× sodium pyruvate (Sigma-Aldrich), ciprofloxacin (10 µg/ml) (AppliChem), human recombinant interleukin-2 (rIL-2; 100 IU/ml), and human rIL-15 (50 ng/ml) (Miltenyi Biotec). CD8$^+$ T cell clones were stimulated in complete RPMI 1640/human serum (HS) medium containing phytohemagglutinin (1 mg/ml) with 1×10$^6$ per ml 35 gray (Gy) irradiated allogeneic peripheral blood mononuclear cells (isolated on FicollPaque Gradient from blood of healthy donors) and 1×10$^6$ per ml 70 Gy irradiated EBV-transformed B cells. Restimulation of clones was performed every 2 weeks. Blood samples were collected and processed following standard ethical procedures (Helsinki protocol), after obtaining written informed consent from each donor and approval by the French Ministry of the Research (transfer agreement AC-2014-2384). Approbation by the ethical department of the French Ministry of the Research for the preservation and conservation of cell lines and clones starting from healthy donor human blood samples has been obtained (authorization no. DC-2018-3223).

HEMn-MP melanocytes were purchased from the American Type Culture Collection. HLA-A2$^+$ human melanoma cell lines used were isolated from metastatic melanoma patients: D10 (provided by G. Spagnoli, Basel, Switzerland), M17 (provided by F. Jotereau, Nantes, France), and EB81-MEL.B and LB2259-MEL.A (provided by P. Coulie and N. V. Baren, Brussels, Netherlands). EBV-transformed B cells (JY) HLA-A2$^+$ were used as sensitive target cells. Melanocytes were maintained in Medium 254 with human melanocyte growth supplement (Thermo Fisher Scientific). Melanoma and JY cells were cultured in RPMI 1640 GlutaMAX supplemented with 10% fetal calf serum (FCS) and 50 µM 2-mercaptoethanol, 10 mM Hepes, 1× MEM-NEAA (Gibco), 1× sodium pyruvate (Sigma-Aldrich), and ciprofloxacin (10 µg/ml) (AppliChem). All cell lines were screened biweekly for mycoplasma contamination using MycoAlert mycoplasma detection kit (Lonza). Melanoma and JY cells were subjected to cell line typing (Microsynth Ecogenics, Switzerland) to authenticate their identity and ensure that no contamination of human origin was detectable.

**FM1-43 incorporation by FACS analysis**

FM1-43 was performed as described previously (60). Melanoma cells were left unpulsed or pulsed with 10 µM antigenic peptide (or lower concentrations when indicated) for 2 hours at 37°C/5%CO$_2$, washed three times, and subsequently transferred to a 96-well U-bottom plate at 2×10$^5$ cells per 25 µl of RPMI 1640 5% FCS/Hepes. CTLs were previously stained with CTV (Invitrogen) for 20 min at 37°C/5% CO$_2$, washed, and added to the target cells at a two CTL/one target cell ratio in 25 µl of RPMI 1640 5% FCS/Hepes. Fifty microliters of FM1-43 (20 µg/ml) (Invitrogen) in RPMI 1640 5% FCS/Hepes was added in 50 µl of cells already contained in the well (FM1-43 final concentration...
was 10 μg/ml). In some experiments, D10 cells were pretreated with 50 μM BAPTA-AM (Invitrogen) and thoroughly washed before conjugation with CTLs. Cells were pelleted for 1 min, 455g and incubated at 37°C/5% CO₂, washed three times, and subsequently transferred to a 96-well U-bottom plate at 20 × 10³ cells per 50 μl RPMI 1640 5% FCS/Hepes. CTLs were previously stained with 0.1 μM 5-chloromethylfluorescein diacetate (CMFDA) for 20 min at 37°C/5% CO₂, washed, and added to the target cells at a two CTL/one target cell ratio in 50 μl of RPMI 1640 5% FCS/Hepes. Cells were pelleted for 1 min, 455g and incubated at 37°C/5% CO₂ for 2, 5, or 15 min. At the end of each incubation time, CTL/melanoma cell cocultures were resuspended and washed in ice-cold PBS containing 2 mM EDTA. Cells were stained with fixable viability dye eFluor 780 (eBioscience) on ice at 4°C for 30 min in FACS buffer (PBS 1% HS, 1% FCS, and 0.01% azide). Samples were acquired using MACS Quant Analyzer 10 (Miltenyi Biotec). Results were analyzed using the FlowJo 10 software.

**CTL CD107a exposure**

CD107a exposure was performed as described previously (60). D10 cells were left un pulsed or pulsed with 0.01 nM to 10 μM of antigenic peptide for 2 hours at 37°C/5% CO₂, washed three times, and subsequently transferred to a 96-well U-bottom plate at 20 × 10³ cells per 50 μl RPMI 1640 5% FCS/Hepes. CTLs were previously stained with 0.1 μM of CMFDA for 30 min at 37°C/5% CO₂, washed, and added to the target cells at a four CTL/one target cell ratio in 100 μl of RPMI 1640 5% FCS/Hepes. Cells were pelleted for 1 min, 455g and incubated at 37°C/5% CO₂ for 2, 5, or 15 min. At the end of each incubation time, CTL/melanoma cell cocultures were resuspended and washed in ice-cold PBS containing 2 mM EDTA. Cells were stained with fixable viability dye eFluor 450 (eBioscience) and with anti-human CD107a PE-Cy7 (10 μg/ml; BD Biosciences) on ice at 4°C for 30 min in FACS buffer. Samples were acquired using MACS Quant Analyzer YVB (Miltenyi Biotec). Results were analyzed using the FlowJo 10 software.

**Cytotoxicity assay**

Cytotoxicity assays were performed as described previously (60). Target cells were left un pulsed or pulsed with 10 μM antigenic peptide for 2 hours at 37°C/5% CO₂, washed three times, and subsequently transferred to a 96-well U-bottom plate at 20 × 10³ cells per 100 μl of RPMI 1640 5% FCS/Hepes. CTLs were previously stained with 0.1 μM CMFDA for 30 min at 37°C/5% CO₂, washed, and added to the target cells at a four CTL/one target cell ratio in 100 μl of RPMI 1640 5% FCS/Hepes. Cells were pelleted for 1 min, 455g and incubated at 37°C/5% CO₂ for 4 hours. In some experiments, CTLs were pretreated with 40 μM monensin (Sigma-Aldrich) and thoroughly washed before conjugation with target cells or pretransfected with perforin siRNA. In additional experiments, melanoma cells were pretreated with 50 μM BAPTA-AM (Invitrogen) and thoroughly washed before conjugation with CTL. In a supplementary set of experiments, D10 cells were previously transfection with a plasmid coding for SytVII shRNA. Before flow cytometry analysis, 0.25 μg of 7-aminomethylcinidin D (BD Biosciences) was added to each sample to measure the percentage of dead target cells. Samples were acquired using MACS Quant Analyzer YVB (Miltenyi Biotec). Results were analyzed using the FlowJo 10 software.

**mRNA coding for CD107a-mApple-SEPfluorin in vitro**

The biosensor was constructed as previously described (60). The vector CD107a-mApple-SEPfluorin was a gift from R. Murrell-Legnado, Sussex University, UK. To produce mRNA coding for CD107a-mApple-SEPfluorin by in vitro transcription, the minimal sequence promoter for T7 RNA polymerase was introduced into the plasmid using Quickchange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s protocol with insert T7 primer F: ATATAAGCAGCTGTGTTTAGTGAACCGTCTAAATCGACTCATTAGAGACCTAGCCATACCG-CTTATTAGACGGTTCACTAAACCAGCTCTGCT-CTAATACGACTCACTATAGAGATCAGGTCTACCGTACC- and insert T7 primer R: CCGTTCAGGTAGCAGGTTTTTGCTATCTATAGTGAGTCGATTAGACGGTTCACTAAACCAGCTCTGCTTTATAT. The linearized plasmid DNA by Not I digestion that contains the T7 RNA polymerase promoter site was then used as template for in vitro transcription with the mMESSAGE mMACHINE T7 Ultra kit according to the manufacturer’s recommendations.

**Transfection of CTL and melanoma cells**

Transfection was performed as previously described (60). A total of 1 × 10⁶ CTLs were washed in Opti-MEM (Gibco) medium and resuspended in 100 μl of Opti-MEM medium (Gibco) and transferred in electroporation cuvettes (0.2 cm, Bio-Rad). One hundred picomoles of three different siRNA-targeting mRNA coding for perforin (ID: 6436 5'-GGCUUAUCUCCAUCAGGt-3’, 6529 5’-GGUCACAAGGCAUCCAc-t-3’, and 6603 5’-GGUGAGAUG-CAACCUAAtt-3’) or nontargeting siRNA was added. Cell suspensions containing 300 pmol of siRNA were electroporated by a pulse of 300 V for 2 ms with a Squarewave Gene Pulser Xcell Electroporation system (Bio-Rad). Electroporated CTLs were transferred into prewarmed culture medium at 37°C with 5% CO₂ and used 16 hours after electroporation. Transfection efficiency was evaluated by flow cytometry.

A total of 8 × 10⁶ D10 cells were washed in Opti-MEM medium and resuspended in 100 μl of Opti-MEM medium and transferred in electroporation cuvettes (0.2 cm). Five or 7 μg of capped and tailed RNA coding for CD107a-mApple-SEPfluorin was added in the cell suspension. After electroporation at 300 V for 2 ms, with a Squarewave Gene Pulser Xcell Electroporation system (Bio-Rad), cells were seeded in prewarmed culture medium at 37°C with 5% CO₂ and used 16 hours after electroporation. Transfection efficiency was evaluated by flow cytometry.

Transfection of 1 × 10⁶ D10 cells with 6 μg of plasmids coding for three different shRNA targeting SytVII or a nontargeting shRNA plasmid (Sigma-Aldrich; shSytVII: TRC0000380620 5’-GTAACCGGAGCACTGGAAGGATGTGTTGCGAAT- CATGTCTCTCCAGTGGTTTTTGG3’, TRC0000318768 5’-CGCAGCCTGAAATGCGAGGATGTGTTGCTCAAAGCCCGGAACCTCAAAGCTGAGCTTTGAGGTTCGCGGCTTGTTAGTTTTTGG3’, and control: no specific target SHC002: 5’CCGGCAACAAAGATGAGACGACCACACTCGGTGTTGCTCTACATTTTGG3’) was performed using Lipofectamine 3000 Reagent (Sigma Life Science) according to the manufacturer’s recommendations. Transfected cells were selected by puromycin treatment.

**Transfection of melanocytes**

As previously described (60), the vector pClpA102-G-HLA-A2_GFP was a gift from T. F. Gregers, Oslo University, UK (Addgene, 85162). To produce mRNA coding for HLA-A2_GFP, the plasmid was first digested by Xba I and the T7 RNA polymerase promoter site is then used as template for in vitro transcription with the mMESSAGE mMACHINE T7 Ultra kit according to the manufacturer’s recommendations. A total of 8 × 10⁵ HEMn-MP cells washed in Opti-MEM medium were resuspended in 100 μl of Opti-MEM and transferred in electroporation cuvettes (0.2 cm). Three, 5, or 7 μg of capped and tailed
RNA coding for HLA-A2-GFP was added in the cell suspension. After electroporation at 300 V for 2 ms, with a Squarewave Gene Pulser Xcell Electroporation systems (Bio-Rad), cells were seeded in prewarmed culture medium and used 16 hours after electroporation. Transfection efficiency was evaluated by flow cytometry.

**Time-lapse microscopy**

Time-lapse microscopy was performed as previously described (60). D10 cells were pulsed with 10 μM peptide, washed, and seeded at 2 × 10⁴ cells per well on poly-D-lysine–coated 15-well chambered slides (Ibidi) 5 min before imaging. Chambered slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C and constant 5% CO₂ concentration. At the beginning of recording, 8 × 10⁴ CTLs were added. To monitor FM4–64 incorporation in melanoma cell/CTL conjugates, CTLs were previously stained with PKH-67 green fluorescent cell linker (Sigma-Aldrich) for 1 min and washed before addition in the chambered slides. FM4–64 (Invitrogen) at a final concentration of 1 μg/ml was added in the culture medium before starting the acquisition.

To monitor [Ca²⁺]i increase in melanoma cells, D10 cells were preloaded with Fluo-4-AM (5 ng/μl) (Molecular Probes) for 30 min at 37°C with 5% CO₂ and washed before seeding in chambered slides. CTLs were preloaded with Tubulin Tracker Green (Invitrogen) and washed before addition in the chambered slides. Cells with highly compartmentalized Fluo-4-AM signal at baseline or with no detectable Fluo-4-AM signal at baseline were excluded from analysis.

To monitor CD107a exposure in melanoma cells, D10 cells transfected with 5 μg of mRNA coding for CD107a-mApple-SepHluorin construct were used. CTLs were previously stained with ViaFluo 405 or 647 live-cell microtubule stain (Biotium) according to the manufacturer’s recommendations. In some experiments, 5 μM NucView 405 caspase-3 substrate (Biotium) was added in the culture medium. In a set of experiments, transfected D10 cells were pretreated with 50 μM BAPTA-AM for 2 hours at 37°C with 5% CO₂ (Invitrogen).

All time-lapse microscopy experiments were acquired on a spinning-disk microscope (Nikon) running on MetaMorph software. In all experiments, cells were in RPMI 1640 5% FCS medium within a temperature-controlled chamber maintained at 37°C and constant 5% CO₂ concentration. A sCMOS Hamamatsu ORCA-Flash 4.0 V3 camera or an emCCD Evolve camera (Photometrics) was used for acquisitions. Image analysis was performed using ImageJ software.

**Western blot analysis of SytVII expression**

Western blots were performed as previously described (60). A total of 2.5 × 10⁶ transfected D10 cells washed in cold PBS were diluted in lysis buffer (25 mM tris hydrochloride, 150 mM sodium chloride, 1% NP-40, and complete protease inhibitor cocktail). After 5 min on ice lysis extracts were centrifuged 14,000g for 15 min to pellet DNA. The supernatant was collected. Reduced 10% SDS gels were run and blotted onto a nitrocellulose membrane. After blocking with 3% nonfat dry milk in tris-buffered saline with Tween 20 (TBST) for 1 hour, the membrane was incubated with rabbit anti-SytVII antibody (Ab) (10 μg/ml) (Abcam) overnight at 4°C followed by 2 hours of incubation with anti-rabbit immunoglobulin G horseradish peroxidase (HRP) (SouthernBiotech). Anti-actin Ab was used as endogenous control for normalization (after stripping procedure of the membranes). The blots were developed using ECL Detection Reagent (GE Healthcare). Western blot images were acquired using ChemiDoc MP System (Bio-Rad).

**cDNA synthesis and PCR**

cDNA synthesis and PCR were performed as previously described (60). RNA isolation was performed using the RNeasy Mini Kit (Qiagen) and RNA concentration was assessed using the NanoDrop 1000 (Thermo Fisher Scientific) system. RNA was converted to complementary DNA (cDNA) using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies).

The gene expression of SYTVII (Hs01590513_m1) and PRF1 (Hs00169473_m1) was evaluated by RT-qPCR using TaqMan gene expression assays (Applied Biosystems) according to the manufacturer’s recommendations, using a LightCycler 480 System (Roche Life Sciences). All reactions were performed in triplicate and relative gene expression levels were evaluated using the comparative CT (threshold cycle) method (2^ΔΔCT). Glyceraldehyde-3-phosphate dehydrogenase (Hs03929097_g1; sequence: 5’-CAAGAGGAAGA-GAGAGCCCCTCACT-3’) was used as endogenous controls for normalization.

**Bioclinical characteristics of patient cohort**

The melanoma clinical cohort included 42 patients treated for advanced melanoma at the Oncodermatology Department of the Institut Universitaire du Cancer de Toulouse and 38 patients treated at the Centre Hospitalier Universitaire (CHU) de Bordeaux. Patients with available FFPE tumor blocks at the Department of Pathology were included in the analysis; 52 blocks from the Toulouse site and 38 blocks from the Bordeaux site were analyzed. Duplicate samples from the same patients either were obtained at different time points or were from different biopsy sites as indicated in table S1. Patients with insufficient material in the FFPE tumor blocks or with missing clinical annotation were excluded from analysis. Samples in Toulouse were stored at the CRB Cancer des Hôpitaux de Toulouse collection. In accordance with French law, the CRB cancer collection has been declared to the Ministry of Higher Education and Research (DC 2009-989) and a transfer agreement has been obtained (AC-2008-820) after approbation by ethical committees. Samples in Bordeaux were stored at the Cancer Biobank of CHU Bordeaux collection. In accordance with French law, the CRB Cancer collection has been declared to the Ministry of Higher Education and Research (DC 2014-2164) and a transfer agreement has been obtained (AC-2019-3595) after approbation by ethical committees.

**Immunohistochemical multiplex staining**

We visualized CD8, CD107a, and Sox10 by multiplex immunohistochemistry and studied the entire tumor fragment and surrounding microenvironment. The Discovery ULTRA (Ventana Medical Systems, Innovation Park Drive Tucson, Arizona 85755 USA, Roche) was used to automate the staining procedure. After dewaxing, tissue slides were heat pretreated using CC1 (pH 8) buffer (05424569001, Roche). Slides were then stained for multiplex immunohistochemistry using the RUO Discovery Universal procedure (v0.00.0370) in a three-step protocol with sequential denaturation [CC2 buffer (pH 6), at 100°C, 05279798001, Roche] after each step. The chromogen sequence recommended was used. Tissue slides were subsequently incubated using the primary antibodies CD107a (clone D2D11, #9091, Cell Signaling Technology Inc.), CD8 (clone C8/144B, M7103, Agilent Technologies) in Envision Flex dliuent (K800621-2, Agilent Technologies), and Sox10 (clone SP267, 07560389001, Roche). Targets were then linked using the OmniMap anti-rabbit (05269679001, Roche) and OmniMap anti-mouse (05269652001, Roche) HRP-conjugated.
secondary antibodies. Visualization of the different targets was lastly established using the Discovery Silver (07053649001, Roche), Purple (07053983001, Roche), and Yellow (07698445001, Roche) detection kits. Tissue slides were counterstained using Hematoxylin (05277965001, Roche) enhanced by Bluing reagent (05266769001, Roche) and permanently mounted with xylene-based mounting medium (Sakura TissuTek Prisma, Sakura Finetek Europe B.V., Netherlands).

**WSI acquisition**

Bright-field multiplex IHC slides were digitized with a Panoramic 250 Flash II digital microscope (3DHISTECH, Budapest, Hungary) equipped with a Zeiss Plan-Apochromat 20× NA 0.8 objective and a CIS VCC-Fc60FR19CL 4 megapixels CMOS sensor (unit cell size, 5.5 × 5.5) mounted on a 1.6x optical adaptor, to achieve a scan resolution of 0.24 μm/pixel in the final image.

**Computer vision methods**

**In vitro: Analysis of FM4-64 fluorescence intensity across the synapse**

Elliptical masks were manually assigned to the CTLs and target cells at each time point. A 32 × 16 pixel rectangular ROI was automatically assigned lengthwise across the synapse, encompassing both the CTL and target cell sides of the synapse and aligned along the axis of the two cells. Average FM4-64 signal intensity across the width of the synapse was quantified at each point along the length of the ROI, generating a histogram of FM4-64 values. AUC was also calculated for each histogram.

**In vitro: Analysis of synaptic versus distal FM4-64**

An elliptical mask was manually assigned to the target or CTL at each time point. Four rectangular regions of interest were defined automatically: (i) target cell synapse, (ii) target cell distal, (iii) CTL synapse, and (iv) CTL distal, each 30 × 8 pixels. Within each ROI, the pixels in which the CTL and target cell masks overlapped were excluded from analysis, leaving only the area that could be unambiguously attributed to either the CTL or the target. The mean of the activated pixels (in which the signal was greater than the threshold for “noise,” set at 32 fluorescence units) was then calculated.

**Tissue: Slices extraction and analysis**

Before analysis of individual patches, the WSI was divided into seven relevant regions: ITR, ITS-1 to ITS-5, and PTS, illustrated in Fig. 7 (C and D) using the algorithm shown in Fig. 8 (algo. 1, l2). To accomplish this, each WSI was assessed according to the HSV scale and the principal tumor area (corresponding to the yellow area enriched in Sox10 high melanoma cells) delineated. The healthy tissue was delineated in a comparable fashion based on the enrichment of blue/purple, as shown in Fig. 7 (C and D). The PTS was identified at the intersection of the healthy tissue with the dilated tumor using the morphological dilation operator. The PTS was then dilated to extract in the order ITS-1 to ITS-5, with each concentric slice having equivalent thickness. The remaining principal tumor region was designated as the ITR. The whole slide was then split into patches. For each patch, four masks (one for each specific stain) were obtained based on color in the HSV scale. The cytoplasm of melanoma cells was approximated and the number of CD107a+ pixels inside was counted. The function called count_cells was used for both CD8 and melanoma counts. For a given staining mask, count_cells returned both the number of cells and their centroid coordinates. Last, the mean CD107a pixel number per melanoma cell was calculated.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 8; GraphPad) to assess statistical significance of the observed differences between groups. Statistical tests used are mentioned in each figure legend. Nonparametric (Mann-Whitney test or Wilcoxon paired t test) or parametric (unpaired or paired Student’s t test) statistical tests were used to compare differences between unpaired groups (Mann-Whitney test or unpaired Student’s t test) or paired groups (Wilcoxon paired t test or paired t test). Two-way ANOVA was used to analyze differences of two independent variables.
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