Introduction

Lethal hit delivery by cytotoxic T lymphocyte (CTL) is a very sensitive biological function [1]. It has been estimated that target cells displaying as few as 1–10 specific pMHC on their surface can trigger cytotoxicity [2]. More recently, it was shown by time-lapse microscopy that as few as three specific pMHC present at the cellular interface can elicit cytotoxicity [3].

Lethal hit delivery is also a very rapid response. Although T lymphocyte activation to cytokine production is a slow process requiring prolonged TCR occupancy and sustained signalling, cytotoxicity occurs within a few minutes after the initial contact between CTL and target cells, independently of the strength of antigenic stimulation [4–6].

Another distinct feature of CTL biology is that they can kill multiple target cells, either simultaneously or serially by recycling from one target to another [5, 7–9]. This property makes cytotoxicity even more efficient, because each individual CTL can annihilate more than one target even in conditions that are not sufficient to activate cytokine production.

Intriguingly, the paradigm of exquisitely efficient CTL does not hold for immune-surveillance against solid tumours. Indeed, tumour-specific CTL expand at relatively high frequency in cancer patients and infiltrate malignant tissues [10, 11]. However, the effector function of these naturally occurring CTL or of CTL induced by immune-therapy protocols is often insufficient to achieve clinical remission [11, 12].

Several molecular mechanisms account for tumour escape from CTL immune-surveillance. Effector CTL generation can be impaired by the low immunogenicity of tumour antigens and low expression of co-stimulation signals [13]. Once generated, effector CTL must overcome additional barriers for successful...
tumour immune-surveillance. On one hand, the tumour microenvironment can be enriched in immune-suppressive cytokines and/or metabolic factors (e.g. TGF-β, IL-10, indoleamine 2, 3-dioxygenase, arginase-1 and nitric-oxide synthase 2) [14, 15]. On the other hand, tumour cells can modulate the expression of surface molecules: (i) to avoid recognition by CTL (e.g. by down-regulating Class I MHC molecules) [16]; (ii) to impair the binding of lytic granules on their surface [17]; (iii) to counter-attack CTL aggression (e.g. by expressing FasL on their surface) [18]. Regulatory CD4+CD25+ cells are also enriched in tumour infiltrates and can further contribute to inhibit CTL effector function [19].

Additional mechanisms of resistance include intra-cellular effectors that in tumour cells ‘defuse’ the lytic potential of CTL. Among these, granzyme inhibitors of the serpin family have been thoroughly characterized [20].

The above findings indicate that failure of CTL-mediated tumour elimination mainly depends on the difficulty to generate effector cells and to have these cells appropriately activated in the tumour microenvironment. Accordingly, the lytic function of ex vivo isolated CD8+ cells from tumour-infiltrating lymphocytes (TIL) is impaired in human and mouse [21, 22]; furthermore, freshly isolated CD8+ TIL exhibit defects in adhesion and in proximal TCR signalling [23, 24].

An additional cause for CTL failure may rely in defective cognate interaction with tumour cells. Surprisingly, although CTL/target cell interaction has been thoroughly investigated both in vitro and in vivo, most of the information available on human CTL biology comes from studies using conventional target cells, such as Epstein-Barr virus (EBV)-transformed B cells and myelomonocytic cell lines. We therefore miss a detailed description of the different steps of CTL/tumour cell interaction that would be instrumental to better define at which level(s) CTL function is defective.

We have examined cytotoxicity exerted by CMV-specific or Melan-A/MART-1-specific CTL conjugated with HLA-A2+ cells derived from solid tumours or with conventional targets pulsed with antigenic peptide.

Our results show that CTL underwent comparable activation and efficiently polarized their lytic machinery towards target cells when conjugated with melanomas or conventional targets, yet tumour cells were resistant to CTL-mediated cytotoxicity. Time-lapse microscopy experiments demonstrated that melanoma cells endure lytic granule delivery for a prolonged time before undergoing apoptosis. The observed ‘delayed’ lysis of tumour cells may constitute a mechanism to reduce CTL efficiency in controlling the growth of solid tumours in vivo.

Materials and methods

T cells and APC
The HLA-A2-restricted T cell line (CMVpp65) specific for the human cytomegalovirus protein pp65 peptide NLVPMVATV [6] and the HLA-A2-restricted T cell clone IFN-DC#1 specific for the Melan-A/MART-1 peptide ELAGIGILTV [25] were used.

The following HLA-A2+ cell lines were used as target cells: EBV-transformed B cells (JY and LG2); T2 (T cell leukaemia/B cell line hybrid defective in TAP1/TAP2); K562-A2 (myelogenous leukaemia cell line K562 transduced with an HLA-A2-encoding plasmid, kind gift from Dr. P. Le Bouteiller, Toulouse, France); HBL and D10 cells (isolated from metastatic melanoma patients, kindly provided by Dr. G. Spagnoli, Basel, Switzerland); M17, M44 and M113 melanoma lines (kindly provided by Dr. F. Jotereau, Nantes, France); MCF-7 mammary tumour line (kind gift from Dr. F. Antunes, Lisboa, Portugal); HepG-2 hepatocarcinoma cell line.

T cells were generated and maintained as described [5, 6]. Target cells were cultured in complete RPMI 1640 supplemented with 10% FCS.

FACS analysis and confocal microscopy
The following mAbs were purchased from BD Biosciences (San Diego, CA, USA): anti-HLA-A2 (clone BB7.2), anti-Perforin (clone o1G9), anti-Granzyme B (clone GB11) and anti-active caspase-3 (clone C92–6055). Mouse anti-human Lamp-1 ascites (clone H4A3) were from the Iowa University (Iowa, USA). Intracellular staining was performed as previously described [6]. To distinguish CTL from target cells in the analysis, CTL were loaded with 1 μM DDAO-SE Far Red (Molecular Probes, Invitrogen, France) in RPMI for 15 min. at 37°C, prior conjugation with the target cells.

FACS analyses were performed on a FACScalibur using the CellQuest Pro program (both from Becton Dickinson, San Jose CA, USA).

Microscopy was performed as previously described [6]. CTL/target cell conjugates were co-cultured for 15 min.

Cytotoxic assay
CTL were conjugated at 2:1 E/T ratio, unless indicated, with target cells either unpulsed or pulsed with 10 μM peptide for 4 hrs or 14 hrs, as indicated. CTL were excluded from the analysis by labelling them before conjugation with 1 μM CMFDA (Molecular Probes) for 20 min. at 37°C. Before FACS analysis, 0.125 μg/ml propidium iodide (PI; Molecular Probes) was added to each sample.

Calcein staining assay
DDAO-SE Far Red labelled CTL were conjugated at 2:1 E:T ratio with target cells either unpulsed or pulsed with 10 μM peptide for 4 hrs at 37°C. Cells were then stained with 0.5 μM calcein (Molecular Probes) for 30 min. at RT and analysed by flow cytometry.

Tunel assay
DDAO-SE Far Red-labelled CTL and unlabelled target cells (either unpulsed or pulsed with 10 μM peptide) were conjugated at a 2:1 E:T ratio for 4 hrs at 37°C. Detection of target cells presenting DNA breaks was done using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany), according to the manufacturer’s instructions and were analysed by flow cytometry.
Time-lapse confocal microscopy

CTL were loaded with LysoTracker Red (Molecular Probes) for 45 min. at 37°C in RPMI 5% FCS to visualize lytic granules. Target cells were labelled with 1 μM calcein (Molecular Probes) in RPMI 5% FCS for 30 min. at RT. In some experiments, JY cells were labelled with 0.5 μM Bodipy 630 (Molecular Probes) at 37°C for 15 min. before calcein staining. Target cells were previously pulsed with 10 μM peptide for 2 hrs at 37°C.

Target cells were seeded into microchambers (Lab-Tek Chamber cover-glass, Nunc, France) previously coated with poly-D-lysine (Sigma, France). Fluorescence measurements were done on a Zeiss LSM-510 or on a Leica SP5 confocal microscope at 37°C and 5% CO2, in RPMI 5% FCS. Image sequences of the time-lapse recording were processed with ImageJ software. In some experiments, target cells were loaded with 5 μM Fluo-4 AM (Molecular Probes) for 45 min. at 37°C. Fluo-4 loading was performed in serum-free medium supplemented with 2.5 mM Probenecid plus 0.08% Pluronic F127 (both from Molecular Probes). The green fluorescence emission of Fluo-4 was monitored by time-lapse confocal microscopy [6].

Statistical analysis

Unpaired Student’s t-test using the GraphPad Prism software was used to determine the statistical significance of differences between the groups.

Results

Melanoma cells efficiently activate viral-specific CTL

We have previously shown that when interacting with peptide-pulsed target cells human CTL specific for the immunodominant epitope of the CMV pp65 protein exhibit a low-threshold cytotoxicity and can kill multiple targets simultaneously [5, 6].

In order to investigate how cells derived from solid tumours would behave as targets of these very efficient CTL, we studied two HLA-A2+ cell lines isolated from metastatic melanoma patients (HBL and D10, Fig. S1).

We initially assessed whether these cells would activate CTL to lethal hit delivery. HBL and D10 cells were pulsed with the antigenic peptide and conjugated with CTL to measure the exposure of Lamp-1, a marker of CTL degranulation. As shown in Fig. 1A, CTL exhibited similar extent of Lamp-1 exposure when conjugated with the two melanoma cell lines as compared to conventional JY targets. In addition, measurements of perforin content in CTL after interaction with target cells showed that CTL were similarly
activated to lytic granule release by conventional or melanoma cells (Fig. 1B). Furthermore, JY and melanoma cells induced similar levels of TCR down-regulation and IFN-γ production in CTL (Fig. S2).

Together, the above results indicate that the amount of antigen expressed by all target cells pulsed with 10 μM peptide is largely sufficient to fully activate CTL biological responses. We therefore employed this peptide concentration to test the capacity of CTL to kill tumour cells and to visualize CTL/target cell dynamic interaction.

**Target cells derived from solid tumours are resistant to anti-viral CTL lethal hit delivery**

Having observed that melanoma cells efficiently activate CTL, we investigated their susceptibility to killing by measuring the frequency of target cells that uptake PI after conjugation with CTL [5, 26]. As shown in Figs 2A and S3A, the specific killing of melanoma cells pulsed with 10 μM antigenic peptide was marginal when compared to conventional targets. We also evaluated the capacity of CTL to exhibit cytotoxicity against three other cell lines commonly used as susceptible targets in cytotoxic assays. As shown in Fig. S3B, CTL efficiently killed peptide-pulsed LG2, T2 and K562-A2 cells. This observation ruled out the possibility that JY cells were hypersensitive to CTL-mediated cytotoxicity and validated their use as control cells in our experiments.

Furthermore, the limited capacity of CTL to kill HBL and D10 cells could not be explained by an intrinsic defect of melanoma cells in PI uptake since both melanoma cells became PI-positive after treatment with H2O2, at a concentration known to induce apoptosis (Fig. S3C) [27].

To evaluate the killing of melanoma cells with an independent assay, we measured the uptake of calcein in target cells after conjugation with CTL. These measurements confirmed that melanoma cells are remarkably resistant to CTL-mediated cytotoxicity when compared to conventional targets (Fig. 2B).

Although at 10 μM peptide concentration melanoma cells fully activated CTL (Figs 1 and S2), we reasoned that the lower levels of HLA-A2 expression in melanoma cells (Fig. S1) could somehow result in a defective cytotoxicity. We thus tested whether increasing HLA-A2 expression by IFN-γ treatment would augment the efficiency of killing. As shown in Fig. 2C, melanoma cells with increased HLA-A2 expression remained resistant to CTL attack.

These observations could be extended to a broader panel of HLA-A2⁺ melanomas and to other solid tumours of different histological origin. All tumour cells tested elicited similar degree of CTL degranulation when compared to conventional targets (Fig. S4A) but were significantly resistant to CTL-mediated cytotoxicity (Fig. S4B), validating the use of HBL and D10 cells as cellular models of solid tumours.

To better characterize the HBL and D10 cell resistance to cytotoxicity, we measured the activation of the executioner caspase-3 and the extent of DNA breaks that represent hallmarks of the apoptotic process. As shown in Fig. S5, melanoma cells exhibited a limited activation of caspase-3 and presented considerably less
DNA breaks after interaction with CTL when compared to conventional targets. This limited induction of apoptosis was not due to defective caspase-3 expression by melanoma cells (Fig. S6).

These results indicate that although melanoma cells fully trigger CTL effector function, the tumour cell apoptotic pathway is defectively activated.

Melanoma cell annihilation requires a high effector/target cell ratio and is time-dependent

All together the above observations highlight an intriguing resistance of melanoma cells to cytotoxicity mediated by CTL they have efficiently activated. These in vitro results were obtained using a relatively low E/T ratio (2:1). Because melanoma cells were shown to be killed by tumour-specific CTL in previous studies at high E/T ratios [28, 29], we asked whether the efficiency of melanoma cell killing could be improved by increasing the ratio of CTL/target cell interaction. A prolonged interaction with CTL resulted in increased killing of HBL cells, but had poor effect on D10 cell lysis (Fig. 3B). This observation indicates that, provided more time, CTL may succeed to kill part of the tumour cells, even at low E/T ratio.

Thus, melanoma cells are not intrinsically resistant to CTL cytotoxicity, because increasing the E/T ratio or prolonging the time of cognate interaction may partially revert the melanoma cell resistance to CTL-mediated attack.

Individual melanoma cells receive and endure CTL hits for a prolonged time

Overall, we find that when individual CTL are stimulated by either conventional or melanoma cells, they get similarly activated. Yet,
they are not efficient in killing melanoma targets on a cell per cell basis.

To better characterize the observed CTL 'weakness' in killing melanoma cells, we first monitored the polarization of the lytic machinery towards conventional or melanoma targets by confocal microscopy in fixed conjugates. CTL did not polarize tubulin cytoskeleton (detected by polarization of Microtubule-Organizing Center, MTOC) and perforin granules towards unpulsed JY (Fig. 4A) and HBL cells (Fig. 4C). On the contrary, the majority of CTL interacting with pulsed JY (Fig. 4B) and HBL cells (Fig. 4D) displayed typical assembly of the lytic synapse, polarizing tubulin cytoskeleton and perforin granules towards their targets. To provide a quantitative evaluation of these data, we determined CTL polarization by two independent methods. Firstly, the polarization of the lytic machinery was scored by blinded visual inspection of randomly selected CTL/target cell conjugates. The results of this analysis showed that CTL efficiently polarized their lytic machinery towards peptide-pulsed melanoma cells (Table 1).

Secondly, we measured in individual CTL/target conjugates the distance between MTOC in CTL and the contact site with target cells. This analysis supports the finding that lytic synapses are efficiently formed at the contact site between CTL and melanoma cells (Fig. S7).

We also visualized lethal hit delivery in living CTL/target cell conjugates by time-lapse microscopy. As shown in Fig. S8 and Movies S1 and S2, CTL polarized lytic granules towards JY and HBL cells within a few minutes, as previously reported [4, 5]. Thus, polarization of lytic machinery is not delayed but typically prompted in CTL interacting with melanoma cells.

We then asked whether the lytic granules were actually secreted by CTL and received by target cells. To address this question, we took advantage of previous observations showing that [Ca²⁺]i rapidly increases in target cells receiving lytic granules [5, 8, 26].

Target cells were loaded with Fluo-4, a probe that increases the intensity of green fluorescence emission upon [Ca²⁺]i increase, and visualized by time-lapse microscopy during their interaction with CTL. We measured the fraction of JY and HBL cells (by scoring

### Table 1 Distribution of tubulin and perforin at the CTL-target cell contact site

|       | U |   | P |   |
|-------|---|---|---|---|
| JY    | 10| 102| 72| 96|
| HBL   | 8 | 50 | 68| 124|

n: number of conjugates analyzed; %: percentage of conjugates exhibiting a polarized profile for n = 100%.
110 CTL/JY conjugates and 170 CTL/HBL conjugates from 10 and 11 movies, respectively) undergoing \([\text{Ca}^{2+}]_{i}\) increase after contact with at least one CTL. This analysis showed that all JY and 96.5% HBL cells rapidly underwent \([\text{Ca}^{2+}]_{i}\) increase after CTL contact. In Fig. 5 and Movies S3–S6, a typical result is depicted. Indeed, \([\text{Ca}^{2+}]_{i}\) increased in both JY and HBL cells upon contact with one or more CTL, demonstrating that both targets received the lytic granules. Interestingly, melanoma cells appeared to undergo a more sustained signalling and to resist longer to CTL aggression as compared to JY targets.

We also investigated the time required to detect blebbing in target cells loaded with calcein after initial contact with CTL. By scoring at least 10 independent movies, we found that only ~30% of HBL cells died during the time of recording as compared to ~95% of JY cells (Movie S7 shows an example of resistant HBL cells). On the fraction of cells that died in both populations, we monitored the time required to undergo apoptosis after initial contact with CTL by measuring the time required to detect target cell blebs leading to cell annihilation. Thirty-two CTL/JY conjugates and 31 CTL/HBL conjugates (from at least 8 different movies) were scored. We determined that among susceptible targets, melanoma cells required longer time to be killed (Fig. S9 and Movies S8 and S9).

To have a direct visualization of HBL prolonged resistance to CTL attack, we used time-lapse microscopy to monitor CTL interacting simultaneously with peptide-pulsed JY and HBL targets. As shown in Movie S10, the time required for killing melanoma cells largely exceeded the time required to annihilate conventional targets. In fact, whereas JY cells rapidly died upon interaction with CTL, none of the HBL cells died during the time of recording.

Together these results indicate that although CMV-specific CTL rapidly secrete and deliver lytic granules to peptide-pulsed melanoma cells, on a cell per cell basis CTL are neither rapid nor efficient in killing melanoma cells.

**Melanoma cells are resistant to Melan-A/MART-1-specific CTL**

The anti-viral CTL used throughout our study may not adequately mimic anti-tumour CTL since fundamental differences in TCR affinity/ T cell avidity have been described [30]. We therefore extended our analysis to Melan-A/MART-1-specific CTL.

We first assessed whether melanoma cells would adequately activate CTL degranulation. Indeed, required for killing antigen concentration (10 \(\mu M\)) CTL exhibited similar extent of Lamp-1 exposure when conjugated with melanoma or JY cells (Fig. 6A). Interestingly, when target cells were either unpulsed or pulsed with a low concentration of antigenic peptide (1 nM), Lamp-1 exposure was more pronounced in CTL interacting with melanoma cells (Fig. 6A). This observation suggests that the endogenously expressed Melan-A/MART-1 antigen on melanoma cells is sufficient to trigger CTL degranulation.

To further characterize Melan-A/MART-1-specific CTL activation, we measured TCR down-regulation in CTL interacting either with melanoma or JY cells. As shown in Fig. 6B, JY and melanoma cells (pulsed with 10 \(\mu M\) peptide) induced similar levels of TCR down-regulation in CTL. Having observed that Melan-A/MART-1-specific CTL are activated following interaction with melanoma cells, pulsed with saturating concentrations of the antigenic peptide, we investigated whether CTL exhibit efficient killing of these target cells. As shown in Fig. 6C, melanoma cells were resistant to cytotoxicity exerted by tumour-specific CTL, as they resisted to anti-viral CTL.

These results indicate that independently of the quality of the antigenic stimulus, CTL, although properly and rapidly activated by melanoma cells, behave as inefficient killers.

**Discussion**

Several lines of evidence indicate that the immune system plays a fundamental role in limiting tumour development and progression. Experimental tumours develop more frequently in immune suppressed animal models [31] and in human beings with congenital or acquired immune deficiencies [32]. However, although a relatively high frequency of CTL directed against various tumour antigens in cancer patients has been described, the potency of these naturally occurring anti-tumour CTL responses in vivo is poor. Moreover, therapeutic interventions aiming to specifically enhance these responses have thus far provided unsatisfactory clinical benefits [12, 33]. These observations together with the description of several mechanisms of tumour cell resistance to CTL-mediated cytotoxicity in vivo [22, 34–36] reinforce the notion that CTL responses are rather inefficient in controlling tumour growth.

Failure in tumour immune-surveillance is difficult to reconcile with the notion that CTL are able to rapidly establish functional lytic synapses at the contact site with target cells bearing very low density of antigenic determinants [2, 3, 5, 6]. This raises the question of whether some steps of CTL/target cell interaction and of lytic synapse formation might be defective in CTL facing tumour targets.

In the present work, based on a detailed morphological and functional analysis of CTL/tumour cell interaction, we provide a first stepping-stone to address this challenging question. In our study, CTL specific for viral or tumour antigens underwent similar degree of activation when conjugated with peptide-pulsed melanoma cells or with control targets. Moreover, the efficiency of lytic synapse formation and the time kinetics of lytic granule polarization were similar in CTL interacting with the different targets.

Our analysis of CTL/tumour cell interaction shows that target cell resistance to CTL attack nullifies the rapidity and sensitivity of CTL responses, because on a cell per cell basis melanoma cell annihilation was “delayed”. These results introduce a new ‘time parameter’ in the paradigm of CTL/target cell interaction. Although the sensitivity and rapidity of CTL responses have been thoroughly investigated, little is known about the time required for annihilation of different targets and how this parameter might influence
Fig. 5 HBL cells, similarly to JY targets, receive the lethal hit. Sequences of snapshots depicting Lysotracker red-labelled CTL interacting either with JY or HBL cells pulsed with 10 μM of the pp65 peptide. To detect lethal hit delivery, target cells were labelled with Fluo-4 AM. Fluo-4 staining intensity is shown using a pseudocolour scale in the bottom panels for JY and HBL cells. Data are representative of at least 10 independent experiments. Numbers indicate time in minutes.
the efficiency of CTL-mediated cytotoxicity. Here we show that in conditions of optimal antigenic stimulation, tumour cells resisted to CTL lytic granule secretion for a prolonged time resulting in inefficient killing. Hits were rapidly delivered by CTL to melanoma cells and were received by the tumours, yet tumour cells endured several hits.

These single cell studies were complemented by whole population FACS analyses showing that by increasing the time of

**Fig. 6** Melanoma cells are resistant to Melan-A/MART-1-specific CTL. (A) Lamp-1 surface expression on CTL after 1 hr of conjugation with JY, HBL and D10 cells either unpulsed (U) or pulsed with 1 nM or 10 μM Melan-A/MART-1 peptide is shown. The left-hand panel is a representative result out of three experiments performed in duplicates. Numbers indicate the frequency of Lamp-1-positive cells. The pool of data obtained in three experiments independently performed is shown in the right-hand panel. Results are expressed as mean ± standard deviation. (B) TCR down-regulation in CTL after 4 hrs of conjugation with JY, HBL and D10 cells either unpulsed (U) or pulsed with 1 nM or 10 μM Melan-A/MART-1 peptide is shown. The left-hand panel is a representative result out of three experiments independently performed in duplicates. Numbers indicate the median fluorescence intensity of TCR in CTL. In the right-hand panel is shown the pool of data obtained in three independent experiments. Results are expressed as mean ± standard deviation. (C) Left-hand panel: Frequency of JY, HBL or D10 cells either unpulsed (U) or pulsed (P) with 10 μM Melan-A/MART-1 peptide that incorporated PI after 4 hrs of conjugation with Melan-A/MART-1 CTL. A representative result out of four independent experiments performed in duplicates is shown. Numbers indicate the frequency of PI-positive cells. Right-hand panel: cumulative data from four independent experiments ***P < 0.0001, *P < 0.05.
CTL/target cell interaction cytotoxicity was augmented. Moreover, cytotoxicity was also enhanced when E/T target ratio was increased, in agreement with previous reports [28, 29].

Together these observations indicate that tumour cell annihilation is a cumulative process enhanced either by increasing the number of killers or the time given to each individual CTL to deliver multiple hits to the same target tumour. They also suggest that the known capacity of CTL to secrete quanta of lytic granules towards individual target cells to rapidly annihilate them while sparing lytic potential for further encounters [5, 9] may be useless or even detrimental in the case of immune-surveillance against tumours.

The molecular mechanisms of the observed melanoma cell resistance to CTL aggression are presently elusive. The melanoma cell lines used in this study exhibited low or absent FAS expression (data not shown). In addition, melanoma cells actually received lytic granules and resisted to many of them. Thus, these melanoma cell lines seem to have developed strategies to evade both FAS- and granzyme B-dependent annihilation. Our results allow to exclude potential candidates involved in melanoma cell resistance to CTL attack. Bcl-2, a known anti-apoptotic protein often associated with tumour progression [37] and cathepsin B, whose surface expression was shown to inhibit perforin binding [38], are similarly expressed in melanomas and JY cells (data not shown). In addition, no increased expression of PI-9, a serine protease inhibitor that inactivates granzyme B [20] and of x-linked inhibitor of apoptosis protein (XIAP) known to be over-expressed in some tumours [39, 40], was observed in melanomas as compared to JY cells (Fig. S10).

Recent mouse in vivo imaging studies analysed CTL killing. In a first study, Mempel et al. showed that CTL killing of peptide-pulsed B cells requires ~20 min. [41]. In a recent study, Bream et al. showed that in vivo killing of tumour cells by CTL requires 5–6 hrs [42]. Our study, by comparing killing of human B cells and human tumour cells, is in agreement with these observations and provides a missing link between them.

All in all, our results are relevant to reconcile in vitro data on CTL efficiency with the unsatisfactory outcome of current immunotherapy approaches. In fact, often no positive and direct correlation between the generation of specific CTL and prognosis is observed in clinical trials [11]. This discrepancy may be due to the fact that the fight between the host immune system and cancer cells may result in an immunoeediting process in which the immune system could sculpt the tumours allowing resistant variants to immune-surveillance to emerge [32]. Although activated in vitro as part of clinical trial protocols, CTL may not be efficient against tumour variants that have accumulated mutations allowing prolonged resistance to multiple cytotoxicity hits.

In conclusion, our results contribute to redefine the notion of CTL as highly sensitive and efficient killer cells. On one hand, we show that CTL activation to lytic granule delivery is specific, rapid and sensitive also when CTL interact with melanoma cells: it can be viewed as a ‘digital’ biological process, triggered by a very small number of specific pMHC. On the other hand, our results show that, from the point of view of target cells, annihilation may be either ‘digital’, as is the case of EBV-B cells, or ‘analogue’, as for melanomas in which a cumulative process requiring many killer cells and prolonged contact time is required. Future challenges should aim at experimental and clinical settings that could turn the death of tumour cells into a ‘digital’ process, allowing CTL to be as rapid and efficient when dealing with cancer cells as when facing conventional targets.

Acknowledgements

We thank Loic Dupré, Eric Espinoza, Michael Esquerré and Jocelyne Demengeot for discussion; Nuno Moreno and Gabriel Martins for help with time-lapse microscopy experiments; Giulio Spagnoli, Leonor Parreira and António Coutinho for critical reading of the manuscript. This work was supported by grants from La Ligue Contre le Cancer ‘Equipe Labellisée 2008’. IC is supported by a postdoctoral fellowship (reference SFRH/BDP/23661/2005) from Fundação para Ciência e a Tecnologia, Portugal.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Melanoma cells express HLA-A2. Surface expression of HLA-A2 in JY, HBL and D10 cells. Thin lines correspond to the staining obtained with the isotype control Ab. Numbers indicate the median fluorescence intensity of HLA-A2 staining (bold lines). A representative result of two independent experiments is shown.

Figure S2: JY and melanoma cells induce similar levels of TCR down-regulation and IFN-γ production. (A) Surface TCR expression levels on CTL after 4 hrs of conjugation with JY, HBL and D10 cells either unpulsed (U) or pulsed with 1 nM or 10 μM pp65 peptide. Results are from three independent experiments performed in duplicates. TCR expression levels on CTL conjugated with unpulsed targets were considered to be 100%. (B) IFN-γ staining in CTL after 4 hrs of conjugation, with JY, HBL and D10 cells either unpulsed (U) or pulsed with 1 nM or 10 μM peptide. Results are from two independent experiments performed in duplicates. In A and B, results are expressed as mean ± standard deviation.

Figure S3: Melanoma cells resist CTL-mediated killing. (A) Efficiency of CTL-mediated killing of HBL and D10 melanomas, as compared to JY cells (considered as 100% lysis). Each dot corresponds to an independent experiment performed in duplicates. To determine killing efficiency, the formula [(P+CTL)/P][(U+CTL)/U] was used, where: P and P + CTL are the frequencies of pulsed targets that incorporated PI, when alone or conjugated with CTL, respectively; U and U + CTL are the frequencies of unpulsed targets that incorporated PI when cultured alone or in conjugation with CTL, respectively. ***P < 0.0001. (B) Efficiency of CTL-mediated killing of 3 additional target cells (LG2, T2 and K562-A2) compared to JY (taken as 100% lysis). Each dot corresponds to an independent experiment done in duplicates. Killing efficiency was determined as described in A. **P < 0.01. (C) Frequency of JY, HBL and
D10 cells that incorporated PI after 21 hrs of treatment with 90 μM H2O2, or in control cultures. Results of a representative experiment out of 4 performed in triplicates are shown. Numbers indicate the frequency of PI-positive cells ± standard deviation.

**Figure S4:** Additional melanoma and epithelial tumour cell lines are resistant to CTL-mediated cytotoxicity. (A) Percentage of CTL that expose Lamp-1 at the surface, after 1 hr of conjugation with JY, M17, M44, M113, MCF-7 and HepG-2 cells, either unpulsed (U) or pulsed (P) with 10 μM peptide. Data are from a representative experiment out of 3 performed in duplicates. Numbers indicate the frequency of Lamp-1-positive cells. (B) Frequency of PI-positive JY, M17, M44, M113, MCF-7 and HepG-2 cells either unpulsed (U) or pulsed (P) with 10 μM peptide after 4 hrs of conjugation with CTL. Data are from a representative experiment out of 3 performed in duplicates. Numbers indicate the frequency of PI-positive cells.

**Figure S5:** Defective CTL-mediated induction of apoptosis in melanoma cells. (A) Frequency of JY, HBL and D10 cells either unpulsed (U) or pulsed (P) with 10 μM M peptide expressing active caspase-3, after 1 hr of conjugation with CTL, as determined by flow cytometry. An E/T ratio of 2:1 was used. Data are from a representative experiment out of 3 performed in duplicates. Numbers indicate the frequency of active caspase-3-positive cells. Pooled data obtained in three independent experiments are shown in the right-hand panel. *P < 0.05 and ***P < 0.0001. (B) Frequency of JY, HBL and D10 cells either unpulsed (U) or pulsed with 10 μM pp65 (P) that exhibit DNA breaks, after 4 hrs of conjugation with CTL. One representative result and pooled data from at least four independent experiments performed in duplicates are shown in the left-hand and right-hand panels, respectively. Numbers indicate the frequency of tunel-positive cells. *P < 0.01 and ***P < 0.0001.

**Figure S6:** Melanoma cells express intracellular caspase-3. The expression of caspase-3 in JY and melanoma cells was determined by intracellular FACS staining (bold lines). Thin lines correspond to the isotype control staining. Numbers indicate the median fluorescence intensity of caspase-3 staining. Data are representative of three independent experiments.

**Figure S7:** Statistical analysis of morphological data. Distances between CTL MTTC and the CTL/target contact site were measured using the Zeiss software. An example of measurement of a CTL/pulsed target cell conjugate is shown in the upper panel. In the lower panel, each dot corresponds to a CTL/target conjugate. ***P < 0.001.

**Figure S8:** CTL rapidly polarize their lytic machinery towards HBL cells. Sequences of snapshots of Lysotracker red-labelled CTL interacting with either calcein-labelled JY or calcein-labelled HBL target cells pulsed with 10 μM pp65. A representative experiment out of 6 is shown.

**Figure S9:** ‘Delayed’ apoptosis in melanoma cells. Plotted is the time required for JY and HBL cells to initiate membrane blebbing that precedes cell annihilation after initial contact with CTL, as determined by time-lapse video microscopy. Numbers correspond to the mean time in seconds necessary for the initiation of target membrane blebbing (** **P = 0.0003).

**Figure S10:** Expression of serpin PI-9 and XIAP do not account for resistance of melanoma cells to CTL-mediated killing. (A) Histograms show the intracellular expression of PI-9 in JY, HBL and D10 cells (bold lines). CTL are used as a positive control of PI-9 expression. Thin lines indicate the staining using the isotype control Ab. Numbers indicate PI-9 median fluorescence intensity. Data are representative of three independent experiments. (B) Intracellular expression of XIAP in JY, HBL and D10 cells (bold lines). Hela cells are used as a positive control of XIAP expression. Thin lines correspond to the staining obtained with the isotype control Ab. Numbers indicate XIAP median fluorescence intensity. Data are representative of three independent experiments.

**Movie S1:** Two CTL, loaded with Lysotracker red to visualize lytic granules, are shown during interaction with a JY cell pulsed with 10 μM of pp65 peptide. Lytic granules (red) rapidly polarize towards the target cell labelled with calcein (green). Recording time in minutes is indicated in upper left corner.

**Movie S2:** CTL labelled as in Movie S1 are shown during interaction with HBL targets pulsed with 10 μM peptide. Lytic granules (red) rapidly polarize towards the target cells labelled with calcein (green).

**Movies S3 and S5:** Detection of [Ca^{2+}] increase in a JY cell loaded with Fluoro-4 AM. A CTL labelled with Lysotracker red (red) is shown during its interaction with one JY target pulsed with 10 μM of peptide. Green staining depicts [Ca^{2+}] increase in the target cell. In Movie S5, only Fluoro-4 staining intensity is shown using a pseudocolour scale.

**Movies S4 and S6:** Detection of [Ca^{2+}] increase in HBL target cells loaded with Fluoro-4 AM. CTL labelled with Lysotracker red (red) are shown during its interaction with HBL targets pulsed with 10μM of pp65 peptide. Green staining depicts [Ca^{2+}] increase in the target cell. In Movie S6, only Fluoro-4 staining intensity is shown using a pseudocolour scale.

**Movie S7:** CTL labelled with Lysotracker red (red) are shown during interaction with 10 μM peptide pulsed-HBL cells labelled with calcein (green).

**Movie S8:** Two CTL labelled with Lysotracker red (red) are shown during interaction with 10 μM peptide pulsed-HBL cells labelled with calcein (green).

**Movie S9:** Three CTL labelled with Lysotracker red (red) are shown during interaction with a 10 μM peptide pulsed-HBL cell labelled with calcein (green).

**Movie S10:** CTL labelled with Lysotracker red (red) are shown during interaction with both JY and HBL target cells pulsed with 10 μM peptide. JY cells are loaded with calcein (green) and Bodipy 630 (blue) to distinguish them from calcein-labelled HBL cells. Target cell death is indicated by the loss of the calcein staining.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2008.00586.x (This link will take you to the article abstract).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.
References

1. Valitutti S, Muller S, Dessing M, et al. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J Exp Med. 1996; 183: 1917–21.

2. Sykulev Y, Joo M, Vturina I, et al. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity. 1996; 4: 565–71.

3. Purhoo MA, Irvine DJ, Huppa JB, et al. T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol. 2004; 5: 524–30.

4. Stinchcombe JC, Bossi G, Booth S, et al. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity. 2001; 15: 751–61.

5. Wiedemann A, Depoil D, Faroudi M, et al. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. Proc Natl Acad Sci USA. 2006; 103: 10985–90.

6. Faroudi M, Utzny C, Salio M, et al. Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. Proc Natl Acad Sci USA. 2003; 100: 14145–50.

7. Rothstein TL, Mage M, Jones G, et al. Cytotoxic T lymphocyte sequential killing of immobilized allogeneic tumor target cells measured by time-lapse microcinematography. J Immunol. 1978; 121: 1652–6.

8. Poenie M, Tsien RY, Schmitt-Verhulst AM. Sequential activation and lethal hit measured by [Ca2+]i in individual cytotoxic T cells and targets. EMBO J. 1987; 6: 2223–32.

9. Isaaz S, Baetz K, Olsen K, et al. Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. Eur J Immunol. 1995; 25: 1071–9.

10. Romero P, Dunbar PR, Valmori D, et al. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytotoxic T lymphocytes. J Exp Med. 1998; 188: 1641–50.

11. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol. 2005; 175: 6169–76.

12. Boon T, Coulie PG, Van den Eynde BJ, et al. Human T cell responses against melanoma. Annu Rev Immunol. 2006; 24: 175–208.

13. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science. 1993; 259: 368–70.

14. Uttenhove C, Pilotte L, Thate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003; 9: 1269–74.

15. Viola A, Bronte V. Metabolic mechanisms of cancer-induced inhibition of immune responses. Semin Cancer Biol. 2007; 17: 309–16.

16. Hicklin DJ, Marincola FM, Ferrone S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. Mol Med Today. 1999; 5: 178–86.

17. Lehmann C, Zeis M, Schmitz N, et al. Impaired binding of perforin on the surface of tumor cells is a cause of target cell resistance against cytotoxic effector cells. Blood. 2000; 96: 594–600.

18. Hahne M, Rimoldi D, Schrotler M, et al. Melanoma cell expression of Fas(Apo-1)/CD95 ligand: implications for tumor immune escape. Science. 1996; 274: 1363–6.

19. Yamaguchi T, Sakaguchi S. Regulatory T cells in immune surveillance and treatment of cancer. Semin Cancer Biol. 2006; 16: 115–23.

20. Medema JP, de Jong J, Peltenburg LT, et al. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. Proc Natl Acad Sci USA. 2001; 98: 11515–20.

21. Whiteside TL. Immune cells in the tumor microenvironment. Mechanisms responsible for functional and signaling defects. Adv Exp Med Biol. 1998; 451: 167–71.

22. Radoja S, Frey AB. Cancer-induced defective cytotoxic T lymphocyte effector function: another mechanism how antigenic tumors escape immune-mediated killing. Mol Med. 2000; 6: 465–79.

23. Koneru M, Monu N, Schaer D, et al. Defective adhesion in tumor infiltrating CD8+ T cells. J Immunol. 2006; 176: 6103–11.

24. Monu N, Frey AB. Suppression of proximal T cell receptor signaling and lytic function in CD8+ tumor-infiltrating T cells. Cancer Res. 2007; 67: 11447–54.

25. Padovan E, Spagnoli GC, Ferrantini M, et al. IFN-alpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8+ effector T cells. J Leukoc Biol. 2002; 71: 669–76.

26. Koele D, Shi L, Feske S, et al. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity. 2005; 23: 249–62.

27. Del Bello B, Valentini MA, Zumino F, et al. Cleavage of Bcl-2 in oxidant- and cisplatin-induced apoptosis of human melanoma cells. Oncogene. 2001; 20: 4591–5.

28. Topalian SL, Solomon D, Rosenberg SA. Tumor-specific cytolyis by lymphocytes infiltrating human melanomas. J Immunol. 1989; 142: 3714–25.

29. Gervois N, Guilloux Y, Diez E, et al. Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumor peptide interactions. J Exp Med. 1996; 183: 2403–7.

30. McManan RH, Slansky JE. Mobilizing the low-avidity T cell repertoire to kill tumors, Semin Cancer Biol. 2007; 17: 317–29.

31. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001; 410: 1107–11.

32. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoeediting: from immunosurveillance to tumor escape. Nat Immunol. 2002; 3: 991–8.

33. Romero P, Cerottini JC, Speiser DE. The human T cell response to melanoma antigens. Adv Immunol. 2006; 92: 187–224.

34. Gassara A, Messai Y, Gaudin C, et al. The decreased susceptibility of metastatic melanoma cells to killing involves an alteration of CTL reactivity. Int J Oncol. 2006; 29: 155–61.

35. Abouzahr S, Bismuth G, Gaudin C, et al. Identification of target actin content and polymerization status as a mechanism of tumor resistance after cytolytic T lymphocyte pressure. Proc Natl Acad Sci USA. 2006; 103: 1428–33.
36. Radoja S, Saio M, Schaer D, et al. CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis. J Immunol. 2001; 167: 5042–51.

37. Anai S, Goodison S, Shiverick K, et al. Knock-down of Bcl-2 by antisense oligodeoxynucleotides induces radiosensitization and inhibition of angiogenesis in human PC-3 prostate tumor xenografts. Mol Cancer Ther. 2007; 6: 101–11.

38. Balaji KN, Schaschke N, Machleidt W, et al. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. J Exp Med. 2002; 196: 493–503.

39. Kashkar H, Seeger JM, Hombach A, et al. XIAP targeting sensitizes Hodgkin lymphoma cells for cytolytic T-cell attack. Blood. 2006; 108: 3434–40.

40. Kluger HM, McCarthy MM, Alvero AB, et al. The X-linked inhibitor of apoptosis protein (XIAP) is up-regulated in metastatic melanoma, and XIAP cleavage by Phenoxodiol is associated with Carboplatin sensitization. J Transl Med. 2007; 5: 6.

41. Mempel TR, Pittet MJ, Khazaie K, et al. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity. 2006; 25: 129–41.

42. Breart B, Lemaitre F, Celli S, et al. Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. J Clin Invest. 2008; 118: 1390–7.