Dynamic visualization of the whole process of cytotoxic T lymphocytes killing B16 tumor cells in vitro

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Abstract. Cytotoxic T lymphocytes (CTLs) play a key role in adoptive cell therapy (ACT) by destroying tumor cells. Although some mechanisms of CTLs killing tumor cells have already been revealed, the precise dynamic information of CTLs’ interaction with tumor cells is still not known. Here, we used confocal microscopy to visualize the whole process of how CTLs kill tumor cells in vitro. According to imaging data, CTLs destroyed the target tumor cells rapidly and efficiently. Several CTLs surrounded one or more tumor cells, and the average time for CTLs destroying one or more tumor cells in vitro is dozens of minutes only. Our study displayed the temporal events of CTLs’ interaction with tumor cells at the beginning up to the point of killing them. Furthermore, the imaging data presented strong cytotoxicity of CTLs toward the specific tumor cells. These results could help us to well understand the mechanism of CTLs’ elimination of tumor cells and improve the efficacy of ACT in cancer immunotherapy. © The Authors. Published by SPIE under a Creative Commons Attribution 4.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI.

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1 Introduction

Cancer immunotherapy, including the use of monoclonal antibodies, tumor vaccines, checkpoint blockade therapy, and adoptive cell therapy (ACT), is considered a breakthrough in cancer therapy.1–3 ACT is reportedly one of the most efficient therapeutic strategies for melanoma treatments.4 Cytotoxic T lymphocytes (CTLs, also called tumor-specific cytotoxic T cells) play an important role in ACT by eliciting strong antitumor immune response due to their abilities to recognize and destroy tumor cells. When meeting a tumor cell, T-cell receptors (TCRs) expressed on the CTLs could recognize specific tumor antigens on the tumor cell and induce its cytotoxic response.5 The mechanisms of CTLs killing tumor cells unfold in several ways: through the secretion of perforin and granzyme B, through the interaction of FasL and Fas on the cell surface, and through the secretion of perforin and granzyme B, through the interaction of FasL and Fas on the cell surface, and through the destruction of tumor cells18

2 Materials and Methods

2.1 Mice

C57BL/6 female mice (6 to 12 weeks old) were obtained from Hunan Slack King of Laboratory Animal Co., Ltd. (Hunan, China). All of the mice were maintained in a specific pathogen-free barrier facility at Animal Center of Wuhan National...
Cyan fluorescent protein (CFP)-B16,22 tetrameric far-red fluorescent protein (tfRFP)-B1620 and CFP-Hela cells were maintained in our lab, and the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, Beijing, China) containing 1% penicillin-streptomycin (HyClone, Beijing, China) and 10% fetal bovine serum (FBS, HyClone). The cells were grown at 37°C in a humidified incubator with 5% CO₂.

2.2 Cell Cultures

C57BL/6 mice were immunized subcutaneously in both flanks with 2.5 × 10⁶ CFP-B16 (or tfRFP-B16) cells [pretreated with 50 μg/ml mitomycin C (Sigma-Aldrich) for 2 h at 37°C]. Seven days after the first immunization, the mice were immunized again, in the same way as the first time. Seven days after the second immunization, the mice were euthanized, and their spleens were dissected to prepare immunocytes. The spleen-derived cells (1 to 2 × 10⁶ per ml) were cultured in 24-well plates (Corning, Suzhou, China) with RPMI-1640 medium (HyClone, Suzhou, China) and were incubated for 18 h at 37°C in a humidified incubator with 5% CO₂.

2.3 Generation of CFP-B16 or tfRFP-B16 Reactive CTLs In Vitro

To image CTLs’ reaction with B16 tumor cells, CFP-B16 (or tfRFP) cells (5 × 10⁴ per well) were seeded into 35-mm culture dishes with cover glass bottoms (NEST Biotechnology Co. Ltd., Shanghai, China) and were incubated for 18 h at 37°C in a humidified incubator with 5% CO₂. Subsequently, 5 days in vitro-cultured and primed CTLs (or freshly separated splenocytes) were added to the dishes containing CFP-B16 (or tfRFP-B16 and CFP-Hela) cells. Prior to adding to the dishes, the CTLs or splenocytes were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester, CFDA SE (CFSE) following instructions in the standard protocol. After the addition of CTLs (or splenocytes), tumor cells were cocultured for 10 min or 4 h and the fluorescent signals were detected using a confocal laser scanning microscopy (Zeiss 710, Carl Zeiss MicroImaging, Inc., Germany, and Olympus FV1000, Japan) at excitation wavelengths of 405 nm for CFSE-B16 (or CFSE-Hela), 488 nm for CFSE-labeled CTLs or splenocytes, and 561 nm for tfRFP-B16. The 5 × 5 large field images (2125 μm × 2125 μm) were captured using a 20× 0.8 NA objective and the sequential imaging was captured using a 40× 1.4 NA objective.

2.4 Confocal Imaging

To image CTLs’ reaction with B16 tumor cells, CFP-B16 (or tfRFP) cells (5 × 10⁴ per well) were seeded into 35-mm culture dishes with cover glass bottoms (NEST Biotechnology Co. Ltd., Shanghai, China) and were incubated for 18 h at 37°C in a humidified incubator with 5% CO₂. Subsequently, 5 days in vitro-cultured and primed CTLs (or freshly separated splenocytes) were added to the dishes containing CFP-B16 (or tfRFP-B16 and CFP-Hela) cells. Prior to adding to the dishes, the CTLs or splenocytes were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester, CFDA SE (CFSE) following instructions in the standard protocol. After the addition of CTLs (or splenocytes), tumor cells were cocultured for 10 min or 4 h and the fluorescent signals were detected using a confocal laser scanning microscopy (Zeiss 710, Carl Zeiss MicroImaging, Inc., Germany, and Olympus FV1000, Japan) at excitation wavelengths of 405 nm for CFSE-B16 (or CFSE-Hela), 488 nm for CFSE-labeled CTLs or splenocytes, and 561 nm for tfRFP-B16. The 5 × 5 large field images (2125 μm × 2125 μm) were captured using a 20× 0.8 NA objective and the sequential imaging was captured using a 40× 1.4 NA objective.

2.5 Flow Cytometry

Flow Cytometry

CFSE-labeled CTLs were collected from cultured lymphocytes by centrifugation in Histopaque®-1.083 (Sigma-Aldrich) and then labeled with 5 μM CFSE (Invitrogen) for 15 min at 37°C. The CFSE fluorescent signal of the CTLs was analyzed using a FACS-Calibur flow cytometer (Guava EasyCyte 8HT, EMD Millipore Corporation, Germany).

2.6 Data Analysis

Confocal imaging data were presented and analyzed with a ZEN blue (Carl Zeiss MicroImaging, Inc., Germany), a FV10-ASW (Olympus, Japan), an Image J (National Institutes of Health) or an Imaris 7.6 (Bitplane) software.

2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.). For comparisons within three or more groups, the Kruskal-Wallis test was performed and followed by Dunn’s multiple comparison tests. For comparisons of two groups, the two-tailed unpaired t-test was performed. Differences between or within groups are denoted as ns for nonsignificant, * for P < 0.05, ** for P < 0.01, and *** for P < 0.001.

3 Results

3.1 Confocal Imaging of CFP-B16 and tfRFP-B16 and Flow Cytometry Analysis of CFSE-Labeled CTLs

We used a confocal microscopy and a flow cytometer to detect the fluorescent signals of the CFP-B16 and tfRFP-B16 tumor cells and CFSE-labeled CTLs. The imaging results showed that more than 90% of the B16 tumor cells expressed CFP or tfRFP stably [Fig. 1(a)]. The flow cytometry results further confirmed that more than 95% of the CFSE-labeled CTLs had CFSE fluorescent signals [Fig. 1(b)].

3.2 Large Field Imaging of CTLs’ Reactions with CFP-B16 In Vitro

After adding CFSE-labeled CTLs (or splenocytes) to CFP-B16 tumor cells (CTLs: CFP-B16 = 25:1) for 10 min, we used a confocal microscopy to image both the tumor cells and CTLs (or splenocytes). To get more information about the CTLs’ interactions with tumor cells, we chose a large field image method (2125 μm × 2125 μm) to observe more cells [Figs. 2(a) and 2(b)]. Through large-field imaging, CTLs were seen to kill the CFP-B16 tumor cells quickly at the beginning (adding CTLs 10 min later). The survival percentage of CFP-B16 after the addition of CTLs was significantly different compared with the addition of splenocytes or the control group (83.8% versus 100.8% or 100.0%, Figs. 2 and 3). Four hours later, the CTLs successfully killed most of the CFP-B16 tumor cells, and the survival percentage of CFP-B16 decreased to 8.5%. Compared with the group adding CTLs, the survival percentage of CFP-B16 in the group adding splenocytes and the control group remained high (89.5% and 101.0%, Fig. 3). The results suggested that CTLs could quickly and efficiently kill the CFP-B16 tumor cells in vitro.

3.3 Dynamic Visualization of How CTLs Attack CFP-B16 Tumor Cells In Vitro

The whole process of CTLs’ interaction with CFP-B16 tumor cells and later killing them was imaged with real-time sequential
confocal microscopy. As shown in Fig. 4 (and Video 1), a cluster of CTLs around some CFP-B16 tumor cells (CTLs: CFP-B16 = 10:1) interacted with them for about 10 min, then the fluorescent signals of the tumor cells gradually decreased, and finally, the CFP fluorescent signals completely disappeared from the tumor cells, the whole process lasting dozens of minutes. In the previous research, the loss of fluorescent signal of FPs which were stably expressed in the cell could represent the cell death.\textsuperscript{22,25,26} Compared with CTLs, the addition of splenocytes to CFP-B16 tumor cells did not cause the CFP signals of tumor cells to disappear during the entire observation period ($\geq 50$ min; Fig. 5, Video 1). The results suggest that the cytotoxicity of CTLs during the process of destroying tumor cells is strong. Furthermore, imaging data provided a clear picture of the quick and efficient killing process of tumor cells by CTLs through direct visualization.

\subsection*{3.4 Dynamic Imaging of How CTLs Specifically Attack tfRFP-B16 Tumor Cells In Vitro}

We also wanted to study the ability of CTLs to specifically attack tfRFP-B16 tumor cells \textit{in vitro}. So, we used CFP-Hela cells as the negative control tumor cells, which were cocultured with tfRFP-B16 in glass-bottomed dishes; CFSE-labeled tfRFP-B16 tumor cells specific CTLs (CTLs: tfRFP-B16/CFP-Hela = 10:1) were added to the culture. We used confocal microscopy
to observe and analyze the fluorescent signals of tTRFP-B16 and CFP-Hela cells at different times (including before adding CTLs, 10 min, 24 h, and 48 h after adding CTLs). The imaging data showed that there was no difference in survival percentages between the tTRFP-B16 and CFP-Hela before or 10 min after adding CTLs [Figs. 6(a) and 6(b)]. The survival percentage of tTRFP-B16 significantly decreased to 28%, 24 h after adding CTLs; and further decreased to 23%, 48 h after adding CTLs [Figs. 6(a) and 6(b)]. Compared with tTRFP-B16, the survival percentage of CFP-Hela markedly increased [199%, 24 h after, and 165%, 48 h after adding CTLs, Figs. 6(a) and 6(b)]. We also observed how in vitro primed CTLs specifically attacked tTRFP-B16 tumor cells and then successfully eliminated them [Figs. 6(c) and 7, Video 2]. Meanwhile, some CFP-Hela cells that were proximate to the killed tTRFP-B16 cells were not attacked by CTLs and emitted stable fluorescent signals during the whole process of CTLs killing tTRFP-B16 tumor cells [Fig. 6(c)]. All these results suggest that the whole-tumor-cell vaccine we used was able to induce tumor-antigen specific CTLs that had the ability to specifically reorganize and efficiently destroy the target tumor cells.

4 Discussion

In the ACT treatment, the adoptive CTLs play the most important role of successfully eliminating tumor cells. To optimize ACT immunotherapy, it is very important to understand the effector mechanisms of CTLs on the cancer cells in vivo. The molecular mechanisms of CTLs attacking target tumor cells comprise specific antigens of tumor cells that were recognized by TCRs of CTLs, and then (1) the Fas ligand (FasL) of CTLs binding to the FAS receptors of tumor cells and initiation of activation of procaspase-8/10 to induce the apoptosis of tumor cells; (2) the CTLs secretion of perforin to form transient pores on the membrane of target tumor cells to promote quick access of granzymes and then induce apoptosis of tumor cells.27,28 Although these molecular mechanisms of how CTLs recognize and kill tumor cells have been characterized in vitro, but little is known about these processes in the living organism especially in the tumor environment. Besides these molecular properties of CTLs, there are various parameters that affect the efficiency of CTLs to eliminate the cancer cells in the complex tumor microenvironment, including the immune suppression environment blocking CTLs to sufficiently infiltrate into the tumor areas and causing CTLs dysfunction,18,22,26,31,32 as well as recognition abilities of CTLs for the target tumor cells.22,33 Although some correlations have been observed between tumor microenvironment and efficiency of CTLs, the accurate mechanisms of complex parameters that affect the CTLs to induce regression of tumors in vivo are still not known clearly. To obtain in vitro primed and activated CTLs that successfully kill tumor cells after ACT treatment, without undesirable or unexpected effectors, it is necessary to assess the activity and cytotoxicity of CTLs on the target tumor cells in vitro before transferred the CTLs.

The characteristics of killing capability of CTLs include cytotoxicity, efficiency, and specificity. First, we analyzed the cytotoxicity and efficiency by large field imaging and dynamic time-lapse imaging to observe the whole process of how CTLs kill the B16 tumor cells (Figs. 4 and 5, Video 1), including three stages: (1) “binding and scanning,” dozens of CTLs simultaneously binding some CFP-B16 cells at the beginning; (2) “recognition,” the prolonged interactions (lasting for dozens of minutes) between CTLs and tumor cells (the CTLs recognition
of the specific antigens on the tumor cells); and (3) “serial burst killing,” the CFP-B16 cells sequentially losing their fluorescent signals and morphologies and then burst and death in a very short time (for a few minutes). At “serial burst killing” stage, the perforin of CTLs rapidly formed pores on the tumor cell membrane to facilitate the granzymes of CTLs enter the tumor cell to induce apoptosis. This stage lasts for a few minutes.28,34 The large pores formed by perforin on the cell membrane, also allowed the “leakage” of FP of the tumor cell. It might be the reason for the tumor cells quickly losing their fluorescent signals in a short time. It is worth noting that, according to our result, the efficient killing ability of CTLs required the CTLs forming “combat groups” to destroy the target tumor cells. The accumulated CTLs in the tumor areas have been proved to be associated with antitumor efficacy and good survival outcomes.35–38 The mechanisms of a positive correlation between increased CTLs infiltration in various cancer and enhanced antitumor responses have not been elucidated clearly. Here, we give a possible reason that is CTLs sufficiently infiltrate to the tumor areas and form “combat groups” to improve the killing rate to successfully eliminate the tumor cells. Therefore, before ACT, it is necessary to use drugs or other methods to abrogate immunosuppression cells in the tumor areas to promote the infiltration of adoptive CTLs. Prior lymphodepletion has been used before the ACT treatment and proved to effectively enhance the curative effects of ACT.1,4,22

Although some studies presented the serial killing of CTLs on the tumor cells, the dynamic information of specific killing of CTLs on the tumor cells were not clearly revealed. Our study also presented the CTLs specifically recognizing and killing the target tfRFP-B16 tumor cells but not CFP-Hela cells. The imaging data presented that, the CTLs bind and scan with tfRFP-B16 tumor cells [Figs. 6(a) and 6(c)], but do not go to the second (recognition) and third stages (serial burst killing) when they around the CFP-Hela cells. This result was different with our assumption and suggested that the recognition ability might be the most important parameter for the CTLs successfully eliminating the target tumor cells. Enhancing the recognition abilities of target tumor cells of CTLs in vivo could lead to successfully eliminate the tumor cells and be a good way to improve the efficiency of ACT. It might be another reason for positive correlations between increased CTLs infiltration in tumor areas and good antitumor responses.
In this study, we also quantified the efficiency of CTLs' destruction of B16 tumor cells (Fig. 3), which was similar to the results obtained by traditional CFSE and PI dual staining cytotoxicity assays by flow cytometry in our previous research. This finding suggested that this dynamic imaging method could be another useful and easy-to-operate method for evaluation of the activity, efficiency, and specific cytototoxicity of CTLs before ACT treatment. Up to now, although extensive studies focus on the apoptosis of tumor cells death induced by CTLs, nonapoptotic cell death pathways have been considered to exist, such as pyroptosis, necroptosis, or autophagy. It is very important to learn more about heterogeneous modalities of tumor cell death induced by CTLs and figure out their roles in the immunotherapy. Direct visualization results potentially help us to well understand the cytotoxic mechanism of CTLs on the target tumor cells and to develop ACT immunotherapy based on CTLs.

5 Conclusions

In this paper, we directly visualized the whole process of how CTLs kill tumor cells in vitro by large-field and real-time imaging methods. The imaging data presented the rapid, efficient, and specific killing process. The dynamic imaging results showed that the average time taken by CTLs to destroy some tumor cells in vitro was about dozens of minutes, which is much faster than CTLs’ destruction of tumor cells in vivo. According to our specific killing imaging data, CTLs’ recognizing the target tumor cells is the most important step for the CTLs efficient serial killing. Furthermore, the multicolor dynamic imaging of CTLs and tumor cells proved that it could become another effective and easy-to-operate method to assess the abilities of CTLs before ACT.

Disclosure

The authors have no conflicts of interest to declare.

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