Journal of Visualized Experiments

Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58841R1
Full Title:	Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells
Keywords:	apoptosis; cancer cell; CD8+ T cell; cytotoxicity; Fluorescence microscopy; myeloid-derived suppressor cells; tumor-associated macrophages
Corresponding Author:	Takanori Kitamura UNITED KINGDOM
Corresponding Author's Institution:	
Corresponding Author E-Mail:	tkitamur@exseed.ed.ac.uk
Order of Authors:	Takanori Kitamura
	Dahlia Doughty-Shenton
	Jeffrey W Pollard
	Neil O Carragher
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Edinburgh, UK

TITLE:

Real time detection of *in vitro* tumor cell apoptosis induced by CD8$^+$ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells

AUTHORS AND AFFILIATIONS:

Takanori Kitamura[1,2*], Dahlia Doughty-Shenton[3*], Jeffrey W. Pollard[2], Neil O. Carragher[3,4]

[1] Royal (Dick) School of Veterinary Studies and Roslin Institute, The University of Edinburgh, Edinburgh, UK

[2] MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, UK

[3] Edinburgh Phenotypic Assay Centre, The University of Edinburgh, Edinburgh, UK

[4] Cancer Research UK Edinburgh Centre, MRC Institute of Genetics, Molecular Medicine, The University of Edinburgh, Edinburgh, UK

[*] Equal contribution

Corresponding authors:

Takanori Kitamura

tkitamur@exseed.ed.ac.uk

Neil O. Carragher

N.Carragher@ed.ac.uk

Email Addresses of Co-authors**:**

Dahlia Doughty-Shenton (D.Shenton@ed.ac.uk)

Jeffrey W. Pollard (Jeff.Pollard@ed.ac.uk)

KEYWORDS:

apoptosis; cancer cell; CD8$^+$ T cell; cytotoxicity; fluorescence microscopy; myeloid-derived suppressor cells; tumor-associated macrophages

SUMMARY:

We describe here a protocol to investigate cytotoxicity of pre-activated CD8$^+$ T cells against cancer cells by detecting apoptotic cancer cells *via* real-time microscopy. This protocol can investigate mechanisms behind myeloid cell-induced T cell suppression and evaluate compounds aimed at replenishing T cells *via* blockade of immune suppressive myeloid cells.

ABSTRACT:

Potentiation of the tumor-killing ability of CD8$^+$ T cells in tumors, along with their efficient tumor infiltration, is a key element of successful immunotherapies. Several studies have indicated that tumor infiltrating myeloid cells (e.g., myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs)) suppress cytotoxicity of CD8$^+$ T cells in the tumor microenvironment, and that targeting these regulatory myeloid cells can improve immunotherapies. Here, we present an in vitro assay system to evaluate immune suppressive

effects of monocytic-MDSCs and TAMs on the tumor-killing ability of $CD8^+$ T cells. To this end, we first cultured naïve splenic $CD8^+$ T cells with anti-CD3/CD28 activating antibodies in the presence or absence of suppressor cells, and then co-cultured the pre-activated T cells with target cancer cells in the presence of a fluorogenic caspase-3 substrate. Fluorescence from the substrate in cancer cells was detected by real-time fluorescence microscopy as an indicator of T-cell induced tumor cell apoptosis. In this assay, we can successfully detect the increase of tumor cell apoptosis by $CD8^+$ T cells and its suppression by pre-culture with TAMs or MDSCs. This functional assay is useful for investigating $CD8^+$ T cell suppression mechanisms by regulatory myeloid cells and identifying druggable targets to overcome it via high throughput screening.

INTRODUCTION:

It is known that $CD8^+$ T cells can eliminate tumor cells when they exert their full cytotoxicity. After activation of the T cell receptor (TCR), $CD8^+$ T cells proliferate and differentiate into cytotoxic effector cells. The expanded and activated $CD8^+$ T cells secrete cytotoxic granules, including perforin and granzymes, that are transferred into target cells and initiate various lytic pathways such as caspase-3 mediated apoptosis[1]. $CD8^+$ T cells can also induce tumor cell apoptosis by activating receptors on target cells, such as receptors for tumor necrosis factor-α (TNF-α), first apoptosis signal ligand (FasL), or TNF-related apoptosis-inducing ligand (TRAIL). Furthermore, the activated $CD8^+$ T cells secrete interferon-γ (IFN-γ) that can suppress tumor cell proliferation and increase the sensitivity of tumor cells to $CD8^+$ T cells *via* the up-regulation of FasL receptor[1]. Given the potential for $CD8^+$ T tumor killing ability, several strategies to boost their cytotoxicity (e.g., checkpoint inhibitors, cancer vaccination, and adoptive transfer of chimeric antigen receptor (CAR) expressing T cells) have been established and shown significant therapeutic effects on certain types of cancer[2]. However, accumulating evidence suggests that tumor-infiltrating immune cells such as regulatory T cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) can suppress $CD8^+$ T cell functions and restrict efficacy of immunotherapies[3–5]. To improve such immunotherapies, it is important to understand how immune suppressor cells limit $CD8^+$ T cell cytotoxicity. The identification of $CD8^+$ T cell suppression mechanisms as well as druggable targets to overcome it, will require the development and utilization of in vitro assays.

The gold standard method of measuring $CD8^+$ T cell cytotoxicity is the chromium release assay in which the release of the radioactive probe (^{51}Cr), from target cells that are lysed by $CD8^+$ T cells, is determined[6]. However, this assay has several drawbacks including relatively low sensitivity, high background, inability to detect early apoptotic events, hazardous disposal problems, and limited compatibility with automated liquid handling and detection to support higher throughput applications. Another common method is flow cytometric analyses in which apoptosis of target tumor cells is detected by annexin V binding[7]. In this assay, it is possible to detect other parameters such as target cell death using propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) and effector cell activation indicated by CD107a or CD69 expression, in addition to the apoptosis in target cells[7]. However, this assay requires large numbers of suppressor cells compared to the chromium release assay. It also requires the detachment and disaggregation of adherent target cells and this can bias the results. Indeed,

the chromium release assay or flow cytometric assay are not commonly used to investigate suppressor cell effects on T cell functions. Instead, the measurement of T-cell proliferation indicated by dilution of a fluorescent dye (e.g., CFSE) pre-loaded into T cells is frequently used to evaluate the inhibition of $CD8^+$ T cell function by suppressor cells. Detection of IFN-γ production from cultured T cells is another standard method to evaluate the effects of suppressor cells on T cell activation[8,9]. However, the results from these assays do not necessarily correlate to the target cell killing ability of $CD8^+$ T cells.

We present here an alternative functional assay to evaluate effects of suppressor cells, particularly macrophages in metastatic tumors, on the cytotoxicity of $CD8^+$ T cells. This method determines cytotoxicity of $CD8^+$ T cells, pre-cultured with or without the suppressor cells in the presence of anti-CD3/CD28 activating antibodies, by detecting tumor cell apoptosis, indicated by fluorescence from a fluorogenic caspase-3 substrate[6] using automated time-lapse microscopy (**Figure 1**). This protocol has several advantages compared to other methods; it requires only a small number of cells, enables detection of adherent tumor cell death with high sensitivity, can image real-time effector-to-target interaction and is amenable to high throughput screening.

In this protocol, metastasis-associated macrophages (MAMs) and their progenitor monocytic-MDSCs (M-MDSCs) isolated from metastatic tumors in mice are used as suppressor cells. In mouse models of metastatic breast cancer, a distinct population of macrophages characterized as $F4/80^{high}Ly6G^{-}CD11b^{high}Ly6C^{low}$ accumulates in the lung containing metastatic tumors. This macrophage population is infrequently found in the normal lung and thus called metastasis-associated macrophages (MAMs)[10]. In these mouse models, another myeloid cell population, defined as $F4/80^{high}Ly6G^{-}CD11b^{high}Ly6C^{high}$, also accumulates predominantly in the metastatic lung where it gives rise to MAMs[11]. Based on their characteristics, the $CD11b^{high}Ly6C^{high}$ MAM progenitor cells might represent M-MDSCs[12].

PROTOCOL:

All procedures involving mice were conducted in accordance with licensed permission from UK Home Office (P526C60B3). Information about commercial reagents and equipment are listed in the **Table of Materials**.

1. Preparation of target cells that express red fluorescent protein in their nuclei

1.1. Obtain a target mouse cancer cell line from an appropriate source.

NOTE: In this protocol, a highly metastatic derivative of E0771 mouse mammary tumor cells (E0771-LG)[13] are used. Parental E0771 cells originate from C57BL/6 mice[14].

1.2. Thaw and maintain a vial of E0771-LG cells with Dulbecco's Modified Eagles Medium (DMEM) including 10% (v/v) fetal bovine serum (FBS) in a cell culture incubator at 37 °C, 95% humidity, and 5% CO_2.

NOTE: It should be confirmed that cells are negative for mycoplasma. To this end, culture E0771 cells (or cells to be tested) for 2-3 days as described above (in the absence of antibiotics and antimycotics), collect 500 µL of culture medium. Centrifuge the medium at 12,419 x *g* for 60 s to eliminate cell debris and transfer supernatant into new tubes. Determine mycoplasma contamination using a commercially available mycoplasma test kit (refer to **Table of Materials**) and/or PCR[15] following the manufacturer's instructions.

1.3. Seed 5 x 10^3 E0771-LG cells per well into a 12-well plate, and culture the cells with 10% (v/v) FBS-DMEM overnight in an incubator at 37 °C, 95% humidity, and 5% CO_2.

NOTE: If the proliferation rate of target cells is low (population doubling time greater than 36 h), the number of cells can be increased to 1 x 10^4.

1.4. Replace the medium with 1 mL of 10% (v/v) FBS-DMEM including 10 µg/mL polybrene and add 25 µL of lentiviral particles (1 x 10^6 TU/mL) encoding a nuclear restricted red fluorescent protein (mKate2, refer to **Table of Materials**).

1.5. Culture the cells for 24 h in an incubator at 37 °C, 95% humidity, and 5% CO_2.

1.6. Replace the medium with 10% (v/v) FBS-DMEM and culture the cells for 24-48 h in an incubator at 37 °C, 95% humidity, and 5% CO_2.

1.7. Replace the medium with 10% (v/v) FBS-DMEM including 1 µg/mL puromycin when cells start to express red fluorescent protein, and culture the cells until they are 80-90% confluent.

NOTE: Concentration of puromycin will be different between target cell types and should be optimized using un-transfected cells.

1.8. Subculture the surviving cells for 1-3 passages with 10% (v/v) FBS-DMEM including 1 µg/mL puromycin, and cryopreserve stocks in a liquid nitrogen vapor phase storage system until use.

2. Isolation of suppressor cells from the tumors in mice

NOTE: In this protocol, suppressor cells (*i.e.,* MAMs and M-MDSCs) are isolated from the lung containing metastatic tumors established by E0771-LG cells. Conditions for tissue dissociation and cell sorting should be optimized to isolate the cells from different tissues.

2.1. Inject 1 x 10^6 cancer cells (E0771-LG) into the tail vein of syngeneic (C57BL/6), female, 7-10 week old mice.

2.2. After 14 days, isolate the lung containing metastatic tumors and prepare single-cell suspensions from the perfused lungs via enzymatic digestion as previously described[11].

NOTE: In this protocol, four mice are injected with cancer cells and their metastatic lungs are combined to obtain sufficient suppressor cells.

2.3. Incubate the single cell suspensions with anti-mouse CD16/CD32 antibody for 30 min on ice, and stain with fluorescent antibodies to CD45, F4/80, CD11b, Ly6C, and Ly6G (refer to **Table of Materials**) for another 30 min[10,11,13].

2.4. Wash the stained cells once with 1 mL of PBS containing 2% (w/v) bovine serum albumin (BSA), and re-suspend the cell pellet with 500–1000 µL of PBS containing 2% (w/v) BSA.

2.5. Add 3 µM of DAPI, and sort M-MDSCs ($DAPI^{-}CD45^{+}F4/80^{+}Ly6G^{-}CD11b^{high}Ly6C^{high}$) and MAMs ($DAPI^{-}CD45^{+}F4/80^{+}Ly6G^{-}CD11b^{high}Ly6C^{low}$) using a cell sorter (**Supplementary Figure 1**).

NOTE: The threshold of Ly6C level to distinguish MAMs ($Ly6C^{low}$) and M-MDSCs ($Ly6C^{high}$) is based on that of resident alveolar macrophages (RMAC). The purity of the sorted cells is measured via flow cytometry with an expected purity of more than 90%.

2.6. Resuspend the sorted cells with 400 µL of DMEM containing 20% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2 mM L-glutamine, 1% (v/v) non-essential amino acid, 1 mM sodium pyruvate, and 50 nM 2-mercaptoethanol (called enriched-DMEM, E-DMEM).

2.7. Count the number of live cells using the Trypan blue exclusion method[16] and adjust to 2 x 10^6 cells/mL with E-DMEM.

2.8. Keep the cells on ice until use.

3. Isolation of $CD8^{+}$ T cells from the spleen of mice

3.1. Isolate the spleen from a mouse that is syngeneic to the target cancer cell line (*i.e.,* C57BL/6 mice in this protocol) as follows:

3.1.1. Euthanize the animal by CO_2 inhalation.

3.1.2. Place the animal on a clean dissection board and wipe the skin with 70% (v/v) ethanol.

3.1.3. Cut the abdominal skin using scissors to expose the spleen

3.1.4. Isolate the spleen, which is located inferior to the stomach, and place it into a tube containing 5 mL of ice-cold PBS.

3.2. Using the inner plunger of a sterile 5 mL syringe, grind the spleen on a 100 µm cell strainer set on a 50 mL tube.

3.3. Pass the cells through the filter using total 10 mL of PBS.

3.4. Centrifuge the cell suspension at 337 x *g* for 5 min, and aspirate the supernatant.

3.5. Resuspend the cell pellet in 1 mL of PBS containing 2 mM EDTA and 0.5% (w/v) BSA (running buffer) and filter through a 40 µm cell strainer.

3.6. Count the live cell number and adjust to 1 x 10^8 cells/mL using the running buffer.

NOTE: Keep a small aliquot of cells as a pre-enrichment sample for a purity check.

3.7. Enrich for $CD8^+$ T cells using a negative selection kit and a magnetic sorter (refer to **Table of Materials**).

3.7.1. Transfer 1 x 10^8 (1 mL) of the splenocytes cells to a 5 mL polystyrene round-bottom tube.

3.7.2. Add 50 µL of biotinylated antibodies, and incubate at room temperature for 10 min.

3.7.3. Add 125 µL of streptavidin conjugated magnetic beads, and incubate at room temperature for 5 min.

3.7.4. Add 1.325 mL of running buffer, and gently mix by pipetting.

3.7.5. Place the tube into the magnet, and incubate at room temperature for 2.5 min.

3.7.6. Pick up the magnet, and pour the enriched cell suspension into a new tube.

3.8. Resuspend the enriched cells with 200 µL of E-DMEM (i.e., DMEM containing 20% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2 mM L-glutamine, 1% (v/v) non-essential amino acid, 1 mM sodium pyruvate, and 50 nM 2-mercaptoethanol).

3.9. Count the number of live cells and adjust to 2 x 10^6 cells/mL with E-DMEM. Keep the cells at 37 °C in a CO_2 incubator until use.

NOTE: Keep a small aliquot of cells as a post-enrichment sample for a purity check.

3.10. Determine the purity of $CD8^+$ T cells by flow cytometry as follows:

3.10.1. Take 1 x 10^4 cells from pre-enrichment (step 3.6) or post-enrichment (step 3.9) samples and adjust total volume of each to 100 µL using running buffer.

3.10.2. Incubate the single cell suspensions with anti-mouse CD16/CD32 antibody for 30 min on ice, and stain with fluorescent antibodies to CD45, CD3, CD4, and CD8 (refer to **Table of Materials**) for another 30 min.

3.10.3. Wash the stained cells with 500 µL of PBS containing 2% (w/v) BSA, and re-suspend the cell pellet with 500–1000 µL of PBS containing 2% (w/v) BSA.

3.11. Add 3 µM of DAPI, and determine the percentage of $CD3^+CD4^-CD8^+$ cells in the total $CD45^+$ cell population.

4. Activation and expansion of the isolated $CD8^+$ T cells

4.1. Aliquot 1 x 10^5 cells per 50 µL $CD8^+$ T cells (prepared in step 3.9) into wells of a U-bottom 96-well plate.

4.2. Add 1 x 10^5 cells per 50 µL suppressor cells (prepared in step 2.7) or 50 µL of E-DMEM into the wells.

4.3. Prepare activation medium that consists of E-DMEM, 4 x 10^4 U/mL colony-stimulating factor 1 (CSF-1), 240 U/mL interleukin-2 (IL-2), 8 µg/mL anti-mouse CD3ε antibody, and 16 µg/mL anti-mouse CD28 antibody.

NOTE: CSF-1 is not required for T cell activation, but is essential for survival of suppressor cells in this protocol (i.e., MAMs and M-MDSCs). Thus, it is retained in the co-culture of T cells with target cancer cells to maintain consistency in culture conditions. Since CSF-1 is found in nano-molar concentrations in all tissues and is required for monocyte/macrophage viability *in vivo,* this is a physiological context for these cells.

4.4. Add 50 µL of activation medium (step 4.3) and 50 µL of E-DMEM with or without reagents to be tested.

4.5. Place the plate into an incubator at 37 °C, 95% humidity, and 5% CO_2 and culture the cells for 4 days.

5. Setup of co-culture of target cells with pre-activated $CD8^+$ T cells

5.1. Add 30 µL of 1:100 diluted growth factor-reduced soluble basement membrane matrix (**Table of Materials**) into the wells of flat bottom 96-well plate suitable for microscopy, and incubate at 37 °C in a CO_2 incubator for at least 1 h.

5.2. Prepare target cells (i.e., E0771-LG cells expressing nuclear-restricted red fluorescent protein).

5.2.1. Incubate the target cells with 1 mL of 0.05% trypsin/EDTA at room temperature for 1 min, and harvest the cells by gentle pipetting.

5.2.2. Add 9 mL of DMEM including 10% (v/v) FBS, and centrifuge the cell suspension at 337 x *g* for 5 min.

5.2.3. Re-suspend the cells with 500 µL of E-DMEM, and count the number of live cells.

5.2.4. Adjust the density to 2 x 10^4 cells/mL (= 1 x 10^3 cells per 50 µL) by adding E-DMEM, and keep the cells on ice.

5.3. Prepare effector cells (i.e., pre-activated $CD8^+$ T cells).

5.3.1. Resuspend the cells in a well of 96-well plate (step 4.5) thoroughly by pipetting, and transfer the floating $CD8^+$ T cells into a new 1.5 mL tube.

NOTE: MAMs and M-MDSCs tightly adhere to the well and are not detached by the pipetting.

5.3.2. To collect remaining cells, add 200 µL of PBS into the well and transfer into the tube in step 5.3.1.

5.3.3. Wash the cells with 1 mL of E-DMEM once (centrifuge at 337 x *g*) for 5 min, aspirate and discard supernatant), and resuspend them with 100 µL of E-DMEM.

5.3.4. Count the number of live cells (using the trypan blue exclusion method), adjust the density to 1.6 x 10^5 cells/mL (= 4 x 10^3 cells/25 µL), and keep the cells on ice.

5.4. Aspirate the basement membrane matrix from each well of the plate in step 5.1.

5.5. Add 1 x 10^3 cells/50 µL of target cells (step 5.2.4) into each well and mix well.

NOTE: To avoid edge effects due to evaporation of medium, only the inner 60 wells of the 96 well plate should be used for analysis.

5.6. Add 25 µL of E-DMEM including 4 x 10^3 U/mL IL-2 and 10 µM fluorogenic caspase-3 substrate (refer to **Table of Materials**).

5.7. Add 4 x 10^3 cells/25 µL of $CD8^+$ T cells (step 5.3.4) into appropriate wells and mix well.

NOTE: The presence of too many cells in a well makes the analysis more difficult. In this model, a 4:1 effector: target ratio (total cell number 5 x 10^3 cells/well) was optimal, but an 8:1 ratio was suboptimal. Wells containing the following four controls are necessary to aid with data analysis: target cells at the density used for the co-culture wells (1 x 10^3 cells/well) in medium with and without caspase-3 substrate, effector cells (1 x 10^3 cells/well) in medium with and without caspase-3 substrate.

5.8. Add 200 µL of PBS or sterile water into all empty wells (particularly wells on the periphery of the plate) to reduce evaporation of medium from experimental wells.

5.9. Set the plate into a time-lapse fluorescence microscope that is maintained at 37 °C, 95% humidity, and 5% CO_2.

6. Imaging of the cells

NOTE: Detailed image acquisition settings will vary with the microscope and fluorophores used; the following general acquisition parameters should be employed for optimal results.

6.1. Using an appropriate autofocus routine on the microscope, acquire images covering at least 25% of the total surface area in each experimental well of the 96-well plate.

6.2. Set the microscope to acquire images in phase contrast as well as a fluorescent channel suitable for the nuclear-restricted red fluorescent protein (mKate2) and a fluorescent channel suitable for the green fluorogenic activated caspase-3 substrate (with excitation at 488nm) (**Figure 2**).

6.3. Capture images in experimental wells in phase contrast and the 2 fluorescent channels every 1 to 3 h for at least 72 h.

7. Image analysis using image analysis software

NOTE: Detailed image analysis settings will vary with the software used (refer to **Table of Materials**); the following general analysis procedures should be employed for optimal results.

7.1. Determine whether spectral un-mixing is required to separate the fluorescent signal emitted by target cell nuclei from that emitted by apoptotic nuclei and if required, set up an analysis protocol to accomplish this;

7.1.1 View a control well containing only target cells (mkate2-labeled) in medium without caspase-3 substrate in the green fluorescent channel.

7.1.2 Observe whether green fluorescence is being emitted by the red nuclei (nuclei appear green)

7.1.3. If green fluorescence is apparent in the nuclei, access the spectral unmixing control in the imaging software and increase the percentage of red removed from green until the green signal disappears (in our experience, this is usually 6-7% for bright mKate2 fluorescence).

7.1.4 If green fluorescence is not apparent in any nuclei, no spectral unmixing is necessary.

NOTE: This spectral unmixing correction test can also be carried out using a well of target cells in medium containing caspase-3 substrate. However, there is usually a very low level of spontaneous apoptosis in target cells (less than 10%), resulting in a few target cell nuclei emitting green fluorescence due to activation of caspase-3 rather than fluorescence bleed through. True fluorescence bleed through is evident under these conditions when green fluorescence is apparent in all nuclei.

7.1.5 View a control well containing only effector cells (nuclei unlabeled) in medium without caspase-3 substrate in the red and green channel individually.

7.1.6 Observe whether either green or red fluorescence is being emitted by the nuclei. If neither red nor green fluorescence is apparent in the individual channels no spectral unmixing is necessary.

NOTE: In our experience, the mouse $CD8^+$ T cells are not auto-fluorescent and thus no spectral unmixing is necessary for these cells. This spectral unmixing correction test can also be carried out using a well of effector cells in medium containing caspase-3 substrate. However, there is usually some level of spontaneous apoptosis resulting in effector cell nuclei emitting green fluorescence due to activation of caspase-3. True fluorescence bleed through is evident under these conditions when green fluorescence is apparent in all nuclei.

7.2. Use a fluorescence background subtraction method (e.g., TopHat), with relevant parameters for the sample, to resolve the fluorescent objects in both fluorescent channels.

NOTE: In the green channel we used TopHat (nuclei radius = 10.0 µm, green fluorescence threshold = 0.7 green calibration units). In the red channel we used TopHat (nuclei radius = 10.0 µm, red fluorescence threshold = 0.5 red calibration units).

7.3. Use appropriate parameters for edge-splitting to resolve individual fluorescent nuclei.

NOTE: We did not use edge splitting in the green or red fluorescence channel.

7.4. Use images in the red channel from the wells containing only target cells (with caspase substrate) to determine the minimum size of target nuclei.

NOTE: We used 80 μm^2.

7.5. Use images in the green channel from the wells containing only effector cells (with caspase substrate) to determine the average size of apoptotic effector nuclei.

NOTE: Single apoptotic effector nuclei ranged from 40 – 80 μm^2 in these experiments.

7.6. Set up an analysis procedure to count the number of fluorescent target cell nuclei using an appropriate minimum size restriction (e.g., minimum area, minimum diameter) (**Figure 2**, target detection mask).

NOTE: We used a minimum nuclei area (red fluorescence) = 80 μm^2.

7.7. Set up an analysis procedure to count the number of apoptotic nuclei which are larger than the mean size of apoptotic effector nuclei (**Figure 2**, size restricted apoptosis mask).

NOTE: Size filter was set to 80 μm^2 (*i.e.,* only areas of green fluorescence larger than this size were counted, thus excluding *single* apoptotic effector nuclei). Using these parameters increases the accuracy of the analysis in counting apoptotic *target* cell nuclei although some aggregates of apoptotic effector cells may be counted.

7.8. Set up an analysis procedure to count the number of *apoptotic target cells* by counting nuclei where red fluorescent signal (from step 7.6) and size-restricted green fluorescent signal (from step 7.7) significantly co-localize (**Figure 2**, R/G overlap mask).

NOTE: An appropriate co-localization may range from 30% to 100% of the mean size of target nuclei. Overlap size filter was set to 40 μm^2.

7.9. Determine the number of apoptotic target cell nuclei as well as the number of target cell nuclei in the wells for each experimental condition over the entire time course.

8. Data analysis using calculation and graphing software

8.1. Graph the number of apoptotic target cell nuclei obtained in step 7.9 over the entire time course for the experimental wells. If multiple wells were used for each experimental condition, graphically present the results as mean ± standard deviation.

8.2. Calculate the apoptotic fraction of the population of target cells by dividing the number of apoptotic cells in each well by the number of target cells in each well at each time point.

8.3. Graph the results obtained in step 8.2.

8.4. Determine the area under the curve (AUC) for each curve. The peak apoptotic fraction of the population for each curve may also be determined as well as the time point at which the peak occurs, if desired. Determine whether the AUC determined for the experimental conditions are significantly different using appropriate statistical tests.

NOTE: The unpaired t-test with Welch's correction was applied to the AUC results.

REPRESENTATIVE RESULTS:

This method is based on simple co-culture of target cancer cells with effector $CD8^+$ T cells that have been pre-cultured with or without suppressor cells in the presence of anti-CD3/CD28 activating antibodies. It detects $CD8^+$ T cell-induced cancer cell apoptosis over time following co-culture, thus enabling evaluation of effects of suppressor cells on cytotoxicity of $CD8^+$ T cells.

Typically, cancer cells increase green fluorescence in their nuclei following activation of a nuclear-targeting caspase biosensor when these cells make contact with $CD8^+$ T cells that are pre-activated by antibodies in the absence of suppressor cells (**Figure 3**; **Supplementary Movie 1**). Green fluorescence from the caspase substrate was detectable for at least 15 h after apoptosis was initiated. Some spontaneous apoptosis of effector $CD8^+$ T cells was also observed over time even if these cells were cultured in isolation (**Figure 4; Supplementary Movie 2**). However, nuclei sizes of $CD8^+$ T cells are smaller than those of cancer cells and thus apoptotic 'effector' cells can be excluded from apoptotic 'target' cell counts by a size restriction image analysis method (**Figure 2 and Figure 4**). Although some target cancer cells show a small rounded shape without green fluorescence, this does not affect the analysis as these cells are undergoing mitosis rather than apoptosis (**Supplementary Movie 3**), and thus are excluded from apoptotic 'target' cell counts by a red/green overlap mask (**Supplementary Figure 2**). Spontaneous apoptosis of target cancer cells is occasionally found even in the single culture (**Supplementary Movie 3**). However, the co-culture of target cancer cells with pre-activated $CD8^+$ T cells increased tumor cell apoptosis above the levels of spontaneous apoptosis in monoculture of cancer cells (**Figure 4**). Generally, when using an optimal ratio of target cancer cells to effector cells, a peak in the number of apoptotic target cancer cells can be observed (**Figure 5A**). This peak is more distinct when the data is expressed as the apoptotic fraction of the target cell population (**Figure 5B**). In this experiment the basal apoptosis of target tumor cells peaked at 24 h (with apoptotic fraction = 0.08), but $CD8^+$ T cell-induced apoptosis reached a maximum level at 17 h (with apoptotic fraction = 0.66).

We further found that $CD8^+$ T cells pre-incubated with MAMs or M-MDSCs could make contact with target cancer cells but this contact seemed to result in fewer instances of cancer cell apoptosis compared to $CD8^+$ T cells pre-activated without suppressor cells (**Figure 3 and Figure 4**; **Supplementary Movie 4 and Supplementary Movie 5**). Although the $CD8^+$ T cells pre-incubated with the myeloid cells occasionally induced cancer cell apoptosis, there was also some proliferation of target cancer cells that have not been stimulated to undergo apoptosis during the time course of the experiment (**Supplementary Movie 6**). Consistent with these findings, the peak fraction of apoptotic cancer cells cultured with $CD8^+$ T cells pre-incubated with myeloid cells (apoptotic fraction = 0.38 at 23 h for MDSC-E and 0.25 at 20 h for MAM-E) was significantly lower than that of cancer cells cultured with $CD8^+$ T cells which were not pre-incubated with the suppressor cells (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1. A scheme showing the experimental procedure. Naïve splenic $CD8^+$ T cells are cultured with anti-CD3/CD28 activating antibodies with or without metastasis-associated macrophages (MAMs) or monocytic-myeloid-derived suppressor cells (M-MDSCs). After 4 days,

floating $CD8^+$ T cells are collected and co-cultured with target cancer cells in the presence of fluorogenic caspase-3 substrate. Apoptotic cancer cells are detected under real-time fluorescence microscopy. Images shown were acquired using a live cell-imaging platform (refer to **Table of Materials**).

Figure 2. Identification of apoptotic target cells distinct from apoptotic effector cells. Top row: Image acquisition in the red channel allows the identification of target cell nuclei by target detection mask (pink analysis mask). Middle row: Images acquired in the green channel indicate apoptotic effector and target cells. A size restricted apoptosis mask (teal analysis mask; greater than 80 μm^2) allows single apoptotic effector cells to be excluded from the analysis. Bottom row: composite images merged red and green channels with phase contrast image (left) or red/green overlap mask (right). Identification of co-localized, size-restricted green fluorescence with red fluorescence (yellow analysis mask), allows more accurate detection of apoptotic target nuclei (yellow arrowhead) by excluding aggregates of apoptotic effector cells (white arrowheads).

Figure 3. Interaction between $CD8^+$ T cells and cancer cells. Stills from representative time-lapse movies of target E0771-LG_NLR cells (T) co-cultured with effector $CD8^+$ T cells (E) at 4:1 effector/target ratio. MDSC-E and MAM-E indicate effector cells pre-incubated with M-MDSCs and MAMs respectively. Composite images (including images from phase contrast, red and green channels) are shown. Arrowheads are tracking the same cells through the different fields and time points. Yellow arrowheads: a target that associates with effectors and undergoes apoptosis, white arrowheads: a target that associates with effectors but does not undergo apoptosis.

Figure 4. Detection of apoptotic cancer cells. Representative fields extracted from time-lapse movies at 18 h after imaging. Composite images (left; phase contrast, red and green channels) and images from red channel without (middle) or with (right) red/green overlap mask (yellow) are shown. Yellow dots in the right column represent apoptotic cancer cells.

Figure 5. $CD8^+$ T cell-induced cancer cell apoptosis. (**A**) Number of apoptotic cancer cells cultured with effector $C8^+$ T cells at different effector to target ratio (E:T). (**B**) Apoptotic fraction of target cell population. Data are means ± SD. Mean area under the curve (AUC) is also shown. Unpaired *t*-test with Welch's correction was used to analyze the AUC. *P<0.0001 compared to E:T=0:1.

Figure 6. Effects of tumor-infiltrating myeloid cells on cytotoxicity of $CD8^+$ T cells. (**A**) Number of apoptotic cancer cells (target: T) cultured with $C8^+$ T cells (effector: E) at 4:1 of E:T ratio. $CD8^+$ T cells were pre-cultured in the absence (black circle) or presence of monocytic-myeloid-derived suppressor cells (MDSC-E: blue circle) or metastasis-associated macrophages (MAM-E: red circle). Data are means ± SD. (**B**) Apoptotic fraction of target cell population. Data are means ± SD. Mean AUC is also shown. Unpaired *t*-test with Welch's correction was used to analyze the AUC. *P<0.0001 compared to T only, $^{\#}$P<0.0001 compared to E+T.

Supplementary Figure 1. Gating strategy to isolate suppressor cells from the metastatic lung. (**A**) Representative dot plots to isolate monocytic myeloid-derived suppressor cells (M-MDSCs) and metastasis-associated macrophages (MAMs). The threshold of Ly6C level to distinguish MAMs ($Ly6C^{low}$) and M-MDSCs ($Ly6C^{high}$) is based on that of resident alveolar macrophages (RMAC). (**B**) Purity of the sorted M-MDSCs ($CD45^+Ly6G^-CD11b^+Ly6C^{high}$) and MAMs ($CD45^+Ly6G^-CD11b^+Ly6C^{low}$).

Supplementary Figure 2. Representative images of mitotic target cells. Stills from representative time-lapse movies of target E0771-LG_NLR cell mono-culture. Top: composite images including images from phase contrast, red and green channels. Bottom: composite images (red and green channels) with red/green overlap mask.

Supplementary Figure 3. Effects of tumor-infiltrating myeloid cells on proliferation of $CD8^+$ T cells. (**A**) Representative histograms showing dilution of fluorescent labeling with CFSE in $CD8^+$ T cells. Naïve splenic $CD8^+$ T cells were isolated as described in Protocol-3 and labeled with 5 μM of CFSE at 37 °C for 15 min. The labeled T cells were cultured in the presence of IL-2 and anti-CD3/CD28 activating antibodies with or without myeloid cells as described in Protocol 4. After 4 days, green fluorescence in T cells was detected by flow cytometer. (**B**) Division index of $CD8^+$ T cells calculated as previously described[17]. Data are means ± SEM. *$P<0.01$ compared to control, $^{\#}P<0.05$ compared to αCD3/CD28 Ab.

Supplementary Movie 1. Movie of Figures 3 and 4; E+T.

Supplementary Movie 2. Movie of Figures 3 and 4; Effector (E).

Supplementary Movie 3. Movie of Figures 3 and 4; Target (T).

Supplementary Movie 4. Movie of Figures 3 and 4; MDSC-E +T.

Supplementary Movie 5. Movie of Figures 3 and 4; MAM-E +T.

Supplementary Movie 6. Movie of Figures 3 and 4; MAM-E +T (proliferation).

DISCUSSION:

This method is based on two separate co-culture steps: co-culturing $CD8^+$ T cells with potential suppressor cells, and co-culturing the 'pre-conditioned' $CD8^+$ T cells with target tumor cells (**Figure 1**). The first co-culture step is quite similar to that for $CD8^+$ T cell proliferation assays commonly used to determine the effect of suppressor cells on $CD8^+$ T cell function. However, T cell proliferation does not always correlate with their cytotoxicity. For example, we have found that co-culture with M-MDSCs or MAMs increased rather than reduced proliferation of $CD8^+$ T cells in the presence of CD3/CD28 activating antibodies (**Supplementary Figure 3**), whereas these pre-conditioned $CD8^+$ T cells demonstrated reduced cytotoxicity against target cancer cells (**Figure 4, Figure 5, Figure 6**). These results highlight the importance of the evaluation of

functional activity, evidenced by target cancer cell apoptosis, offered by this $CD8^+$ T cell cytotoxicity assay.

In this assay, we have identified that $CD8^+$ T cells requires approximately 15 h of co-culture in order to induce maximum apoptosis of E0771-LG mouse mammary tumor cells (**Figure 5**). This delay might be due to the lag between initial contact of effector cells with targets and accompanying immune synapse formation, as well as time required to induce apoptotic signals in targets as measured by activation of caspase-3 (**Supplementary Movie 1**). We also identified that the number of apoptotic tumor cells reached a plateau after 24 h, which is probably due to the elimination of targets by T cells and/or loss of fluorescent signal from dead cells. This capability to identify the time of peak apoptosis is one major advantage of this assay since determination of an optimal time point is important for appropriate comparisons between different conditions. In our case for example, the difference in cytotoxicity between control $CD8^+$ T cells and MDSC/MAM-educated $CD8^+$ T cells was much larger at 15 - 18 h compared to 72 h (**Figure 5**), and thus an endpoint experiment using a 72 h incubation period would yield misleading results.

This method also enables visualization of real-time effector-to-target cell interaction, which would provide greater insights into the mechanism underlying limited cytotoxicity of $CD8^+$ T cells pre-incubated with suppressor cells. For example, we observed that $CD8^+$ T cells pre-incubated with M-MDSCs or MAMs encountered and interacted with target tumor cells but did not always induce apoptosis (**Supplementary Movie 4, Supplementary Movie 5, Supplementary Movie 6**). Although we did not quantify this event, it would be feasible and interesting to quantify and compare the proportion of encounters and their interaction time in correlation with apoptosis induction. Another major advantage is that this method requires a small number of cells (e.g., 1×10^3 of target and 4×10^3 of effector cells per well). In fact, this protocol can be further miniaturized for the 384-well plate format if desired. Therefore, this assay is suitable for high throughput screening and experiments where cell numbers are limited such as *in vitro* testing using precious cells derived from in vivo or ex vivo samples.

On the other hand, a limitation of the current assay is the presence of significant numbers of dead effector cells in some conditions. In order to increase accuracy in distinguishing apoptosis of target cancer cells from that of effector $CD8^+$ T cells, the nuclei of target cells are labeled and a nuclei size restriction (that excludes effector cells) is applied for data analysis in this assay (**Figure 2**). However, there are some instances where overlay of aggregates of (green) apoptotic $CD8^+$ T cells onto non-apoptotic target cancer cells, which may confound the results. This limitation could be mitigated by use of a dead cell removal column on effector cells prior to co-culture with target tumor cells, assuming sufficient numbers of effector cells are available. With more complex microscopy systems, it may also be possible to reduce the false positive signal by labeling the effector $CD8^+$ T cells with a fluorophore distinct from the target cell nuclei and the fluorogenic caspase-3 substrate.

So far, this protocol has been utilized to investigate the antigen non-specific activation of $CD8^+$ T cells. Although MDSCs and TAMs in the tumor microenvironment suppress T cell functions

through antigen non-specific mechanisms, MDSCs in the peripheral lymphoid tissues suppress T cell responses in an antigen specific manner[18]. To investigate immune suppressive functions of such cell types, an in vitro proliferation assay using $CD8^+$ T cells from OT-1 transgenic mice is commonly used. In this assay, the OT-1 T cells (expressing ovalbumin (OVA) specific T cell receptor) are co-cultured with suppressor MDSCs in the presence of OVA peptides, which is applicable for the first culture in our cytotoxicity assay (i.e*.,* activation of T cells in the presence or absence of suppressors). It is also feasible to manipulate target cancer cells to express OVA, which can induce antigen-specific cancer cell killing by OT-1 T cells. Therefore, the assay will also enable investigation of the MAM/MDSC-mediated suppression of antigen-specific T cell activation. It is also possible to apply the assay to investigate human cells, as activation antibodies against human CD3 and CD28 are commercially available, and a protocol to isolate human TAMs from clinical samples has been established[19].

Collectively, this assay is quite versatile and can be used to examine cytotoxicity of other immune cell types. Currently, in our labs, it is being extended to examine antigen-dependent cell cytotoxicity under various conditions and is also being developed for high throughput screening.

ACKNOWLEDGMENTS:

This work was supported by grants from the Wellcome Trust (101067/Z/13/Z (JWP), 109657/Z/15/Z (TK), 615KIT/J22738 (TK), UK), and the MRC (MR/N022556/1 (JWP, TK), UK). NOC and DDS acknowledge support from the National Phenotypic Screening Centre Phenomics Discovery Initiative and Cancer Research UK (NOC)

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Barry, M., Bleackley, R.C. Cytotoxic T lymphocytes: all roads lead to death. *Nature Reviews in Immunology*. **2,** 401–409 (2002).
2. Durgeau, A., Virk, Y., Corgnac, S., Mami-Chouaib, F. Recent advances in targeting CD8 T-cell immunity for more effective cancer immunotherapy. *Frontiers in Immunology.* **9**, 14 (2018).
3. Tanaka, A., Sakaguchi, S. Regulatory T cells in cancer immunotherapy. *Cell Research.* **27**, 109–118 (2017).
4. Fleming, V. et al. Targeting myeloid-derived suppressor cells to bypass tumor-induced immunosuppression. *Frontiers in Immunology.* **9,** 398 (2018).
5. Cassetta, L., Kitamura, T. Macrophage targeting: opening new possibilities for cancer immunotherapy. *Immunology.* **155,** 285-293 (2018).
6. Chahroudi, A., Silvestri, G., Feinberg, M.B. Measuring T cell-mediated cytotoxicity using fluorogenic caspase substrates. *Methods.* **31,** 120–126 (2003).
7. Zaritskaya, L., Shurin, M.R., Sayers, T.J., Malyguine, A.M. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Review of Vaccines.* **9,** 601–616 (2010).
8. Azimi, M. et al. Identification, isolation, and functional assay of regulatory T Cells. *Immunological Investigation.* **45,** 584–602 (2016).

9. Bruger, A.M. et al. How to measure the immunosuppressive activity of MDSC: assays, problems and potential solutions. *Cancer Immunology Immunotherapy.* (2018). doi: 10.1007/s00262-018-2170-8.
10. Qian, B. et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One*. **4,** e6562 (2009).
11. Kitamura, T. et al. Monocytes differentiate to immune suppressive precursors of metastasis-associated macrophages in mouse models of metastatic breast cancer. *Frontiers in Immunology.* **8,** 2004 (2018).
12. Bronte, V. et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nature Communications*. **7,** 12150 (2016).
13. Kitamura T. et al. CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *Journal of Experimental Medicine.* **212,** 1043–1059 (2015).
14. Ewens, A., Mihich, E., Ehrke, M.J. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Research.* **25,** 3905–3915 (2005).
15. Choppa, P.C. et al. Multiplex PCR for the detection of Mycoplasma fermentans, M. hominisandM. penetransin cell cultures and blood samples of patients with chronic fatigue syndrome. *Molecular and Cellular Probes.* **12,** 301–308 (1998).
16. Strober, W. Trypan blue exclusion test of cell viability. *Current protocols in immunology.* Appendix 3:Appendix 3B (2001)
17. Koyanagi, M., Kawakabe, S., Arimura, Y. A comparative study of colorimetric cell proliferation assays in immune cells. *Cytotechnology.* **68,** 1489-98 (2016).
18. Gabrilovich, D.I., Nagaraj, S. Myeloid-derived-suppressor cells as regulators of the immune system. *Nature Reviews in Immunology.* **9,** 162–174 (2009).
19. Cassetta, L. et al. Isolation of mouse and human tumor-associated macrophages. *Advances in Experimental Medicine and Biology.* **899,** 211-29 (2016).

Figure 1 Kitamura et al.

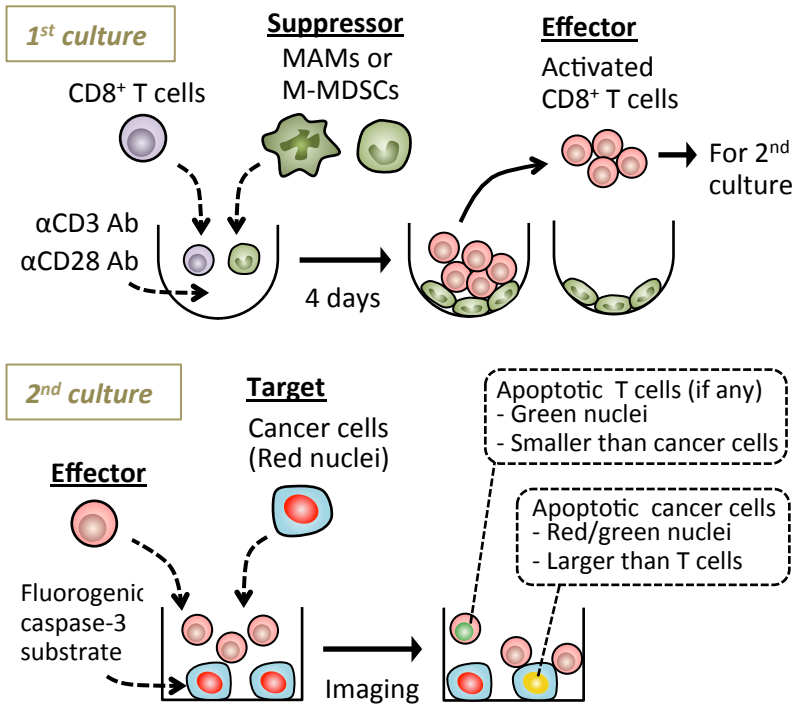


Figure 2 Kitamura et al.

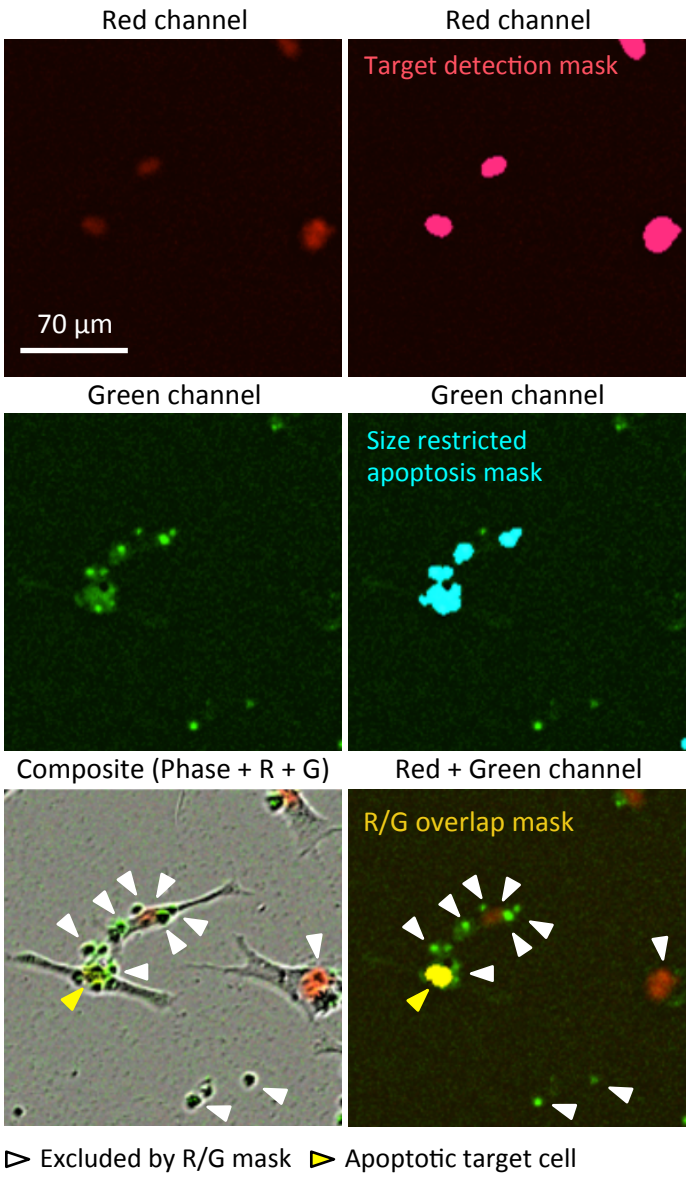


Figure 3 Kitamura et al.

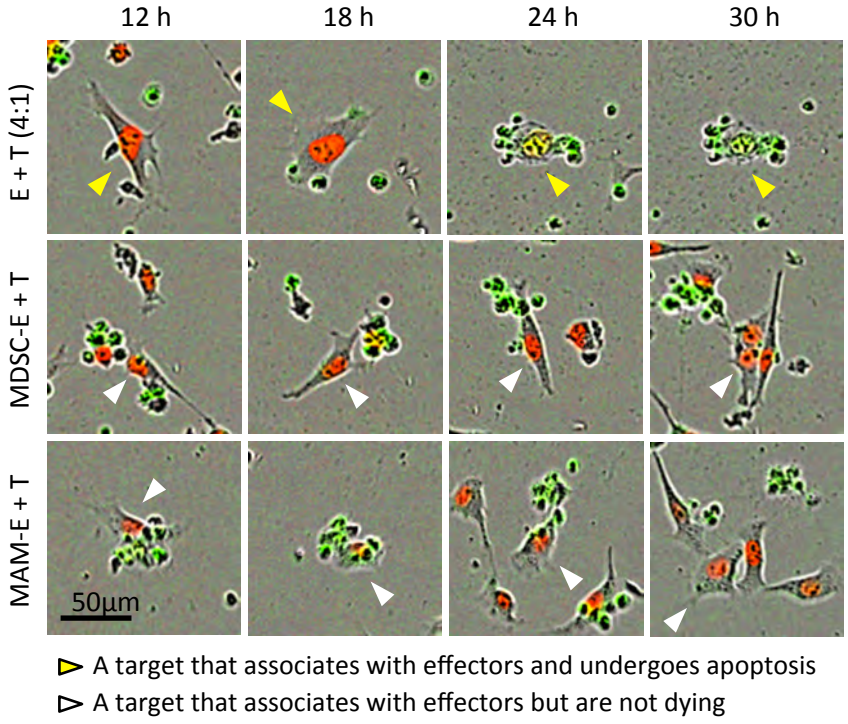


Figure 4 Kitamura et al.

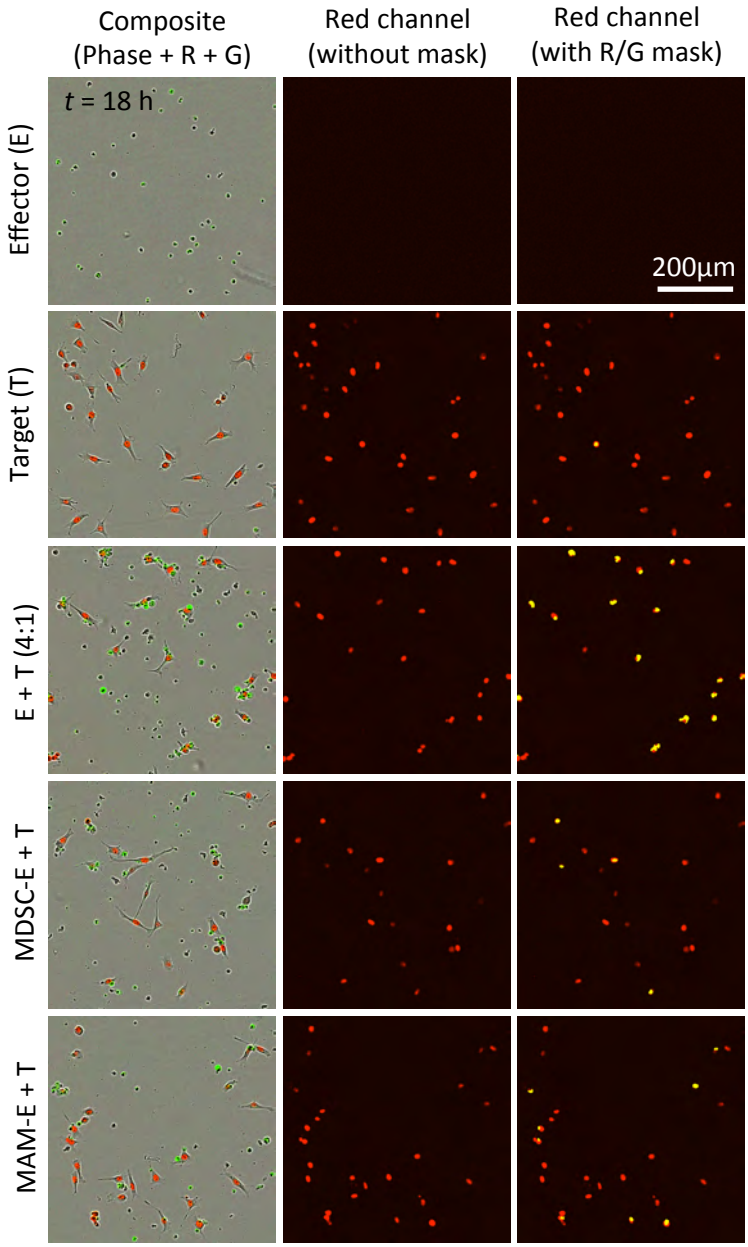


Figure 5 Kitamura et al.

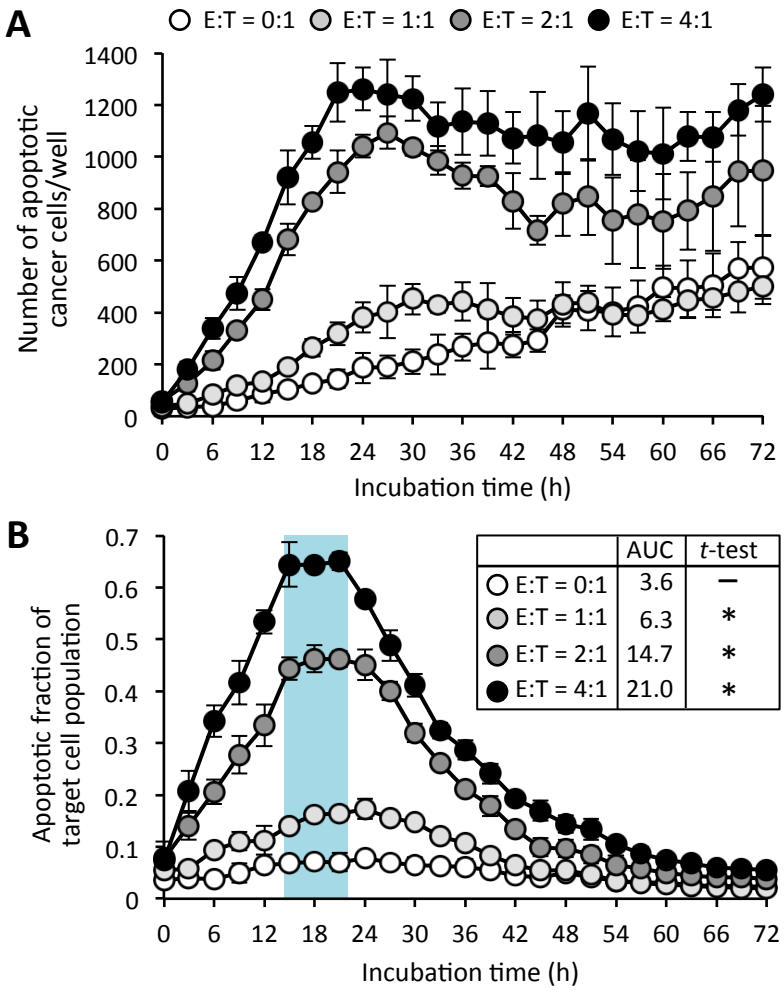
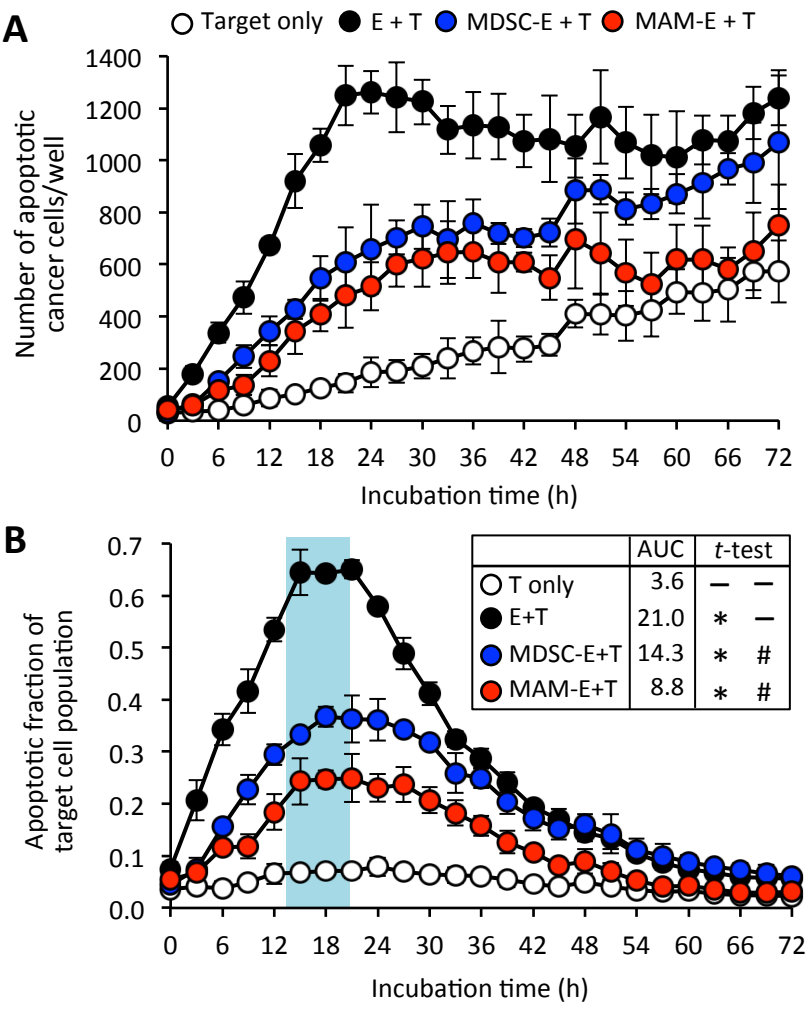
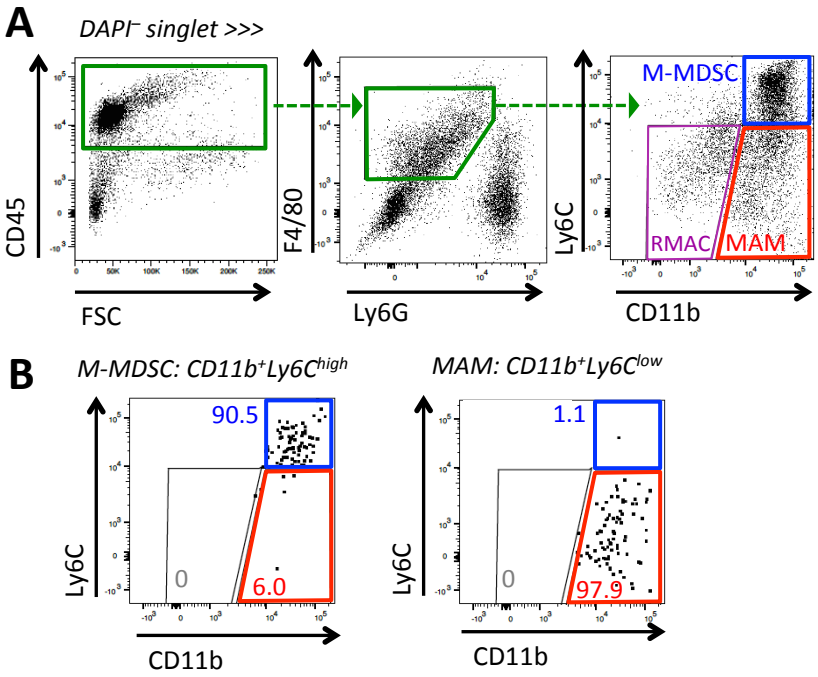


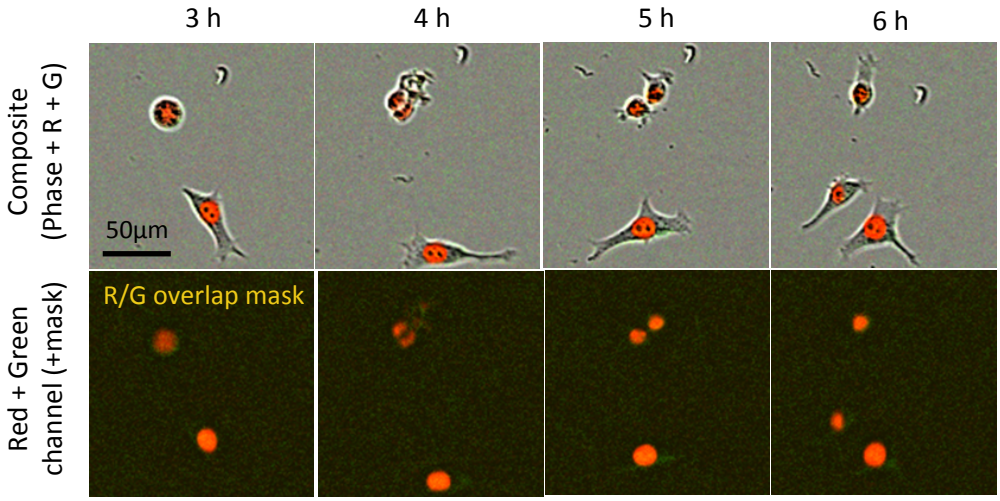
Figure 6 Kitamura et al.



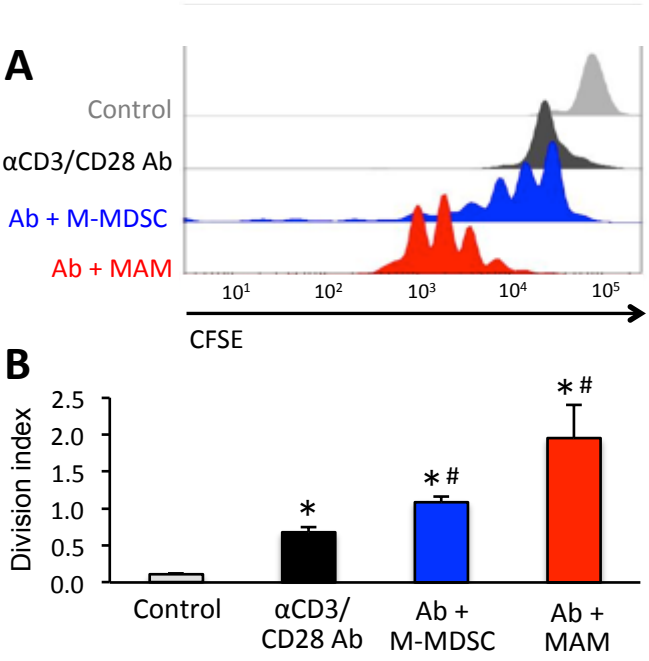
Supplementary Figure 1 Kitamura et al.



Supplementary Figure 2 Kitamura et al.



Supplementary Figure 3 Kitamura et al.



Movie S1

Click here to access/download
Video or Animated Figure
Movie S1_E+T.mp4

Movie S2

Click here to access/download

Video or Animated Figure

Movie S2_Effector.mp4

Movie S3

Click here to access/download
Video or Animated Figure
Movie S3_Target.mp4

Movie S4

Click here to access/download

Video or Animated Figure

Movie S4_MDSC-E+T.mp4

Movie S5

Click here to access/download

Video or Animated Figure

Movie S5_MAM-E+T.mp4

Movie S6

Click here to access/download

Video or Animated Figure

Movie S6_MAM-E+T(proliferation).mp4

Table of Materials

Click here to access/download;Table of Materials;Table of Materials_Rvd.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin EDTA (1X)	Gibco	25300-054	
12-Well Cell Culture Plate	Freiner Bio-One	665-180	
1x PBS	Gibco	14190-094	
2-mercaptethanol	Sigma	M6250-10ML	
5mL Plystyrene Round-Bottom Tube	FALCON	352054	
96-Well Cell Culture Plate (Round Bottom)	Costar	3799	Co-culture of CD8+T cells with sorted myeloid cells
96-well plate (Flat bottom)	Nunc	165305	Co-culture of CD8+T cells with target cells for cytotoxicity assay
AF647 anti-mouse F4/80 Antibody	BIO-RAD	MCA497A647	Clone: CIA3-1, Lot#: 1707, 2 µL/1x10^6 cells
AlexaFluor700 anti-mouse CD8 Antibody	Biolegend	100730	Clone: 53-6.7, Lot#: B205738, 0.5 µL/1x10^6 cells
anti-mouse CD28 Antibody	Biolegend	102111	Activation of isolated CD8+ T cells, Clone: 37.51, Lot#: B256340
anti-mouse CD3e Antibody	Biolegend	100314	Activation of isolated CD8+ T cells, Clone: 145-2C11, Lot#: B233720
APC anti-mouse CD3 Antibody	Biolegend	100236	Clone: 17A2, Lot#: B198730, 0.5 µL/1x10^6 cells
APC/Cy7 anti-mouse Ly6C Antibody	Biolegend	128026	Clone: HK1.4, Lot#: B248351, 1 µL/1x10^6 cells
Bovine Serum Albmin	Sigma	A1470-100G	
Cell Strainer (100µm Nylon)	FALCON	352360	To smash the spleen
Cell Strainer (40µm Nylon)	FALCON	352340	To filter the lung digestion
DAPI	Biolegend	422801	
Dulbecco's Modified Eagle's Medium	Gibco	41966-029	
EasySep Mouse CD8+ T Cell Isolation Kit	StemCell Technologies	19853	
Fetal Bovine Serum	Gibco	10270-106	
FITC anti-mouse CD4 Antibody	Biolegend	100406	Clone: GK1.5, Lot#: B179194, 0.5 µL/1x10^6 cells
Geltrex Ready-to-Use	Gibco	A1596-01	Coating the 96-well plates for cytotoxicity assay
IncuCyte NucLight Red Lentivirus Reagent	Essen BioScience	4476	Lenti viral particules encoding mKate2
IncuCyte ZOOM	Essen BioScience		Detector (fluorescence microscope)
IncuCyte ZOOM 2018A	Essen BioScience		Analysis software
L-Glutamine (100X)	Gibco	A2916801	
Lung Dissociation Kit	Miltenyi	130-095-927	Preparation of single cell suspension from the tumor-bearing lung
MycoAlert Mycoplasma Detection Kit	Lonza	LT07-318	
Non-essential amino acid (100X)	Gibco	11140-035	
NucView488	Biotium	10403	Fluoregenic caspase-3 substrate
PE anti-mouse Ly6G Antibody	Biolegend	127607	Clone: 1A8, Lot#: B258704, 0.5 µL/1x10^6 cells
PE/Cy7 anti-mouse CD11b Antibody	Biolegend	101216	Clone: M1/70, Lot#: B249268, 0.5 µL/1x10^6 cells
Pen Strep	Gibco	15140-122	Penicillin Streptomycin for primary culture of cells
PerCP/Cy5.5 anti-mouse CD45 Antibody	Biolegend	103132	Clone: 30-F11, Lot#: B249564, 0.5 µL/1x10^6 cells
Polybrene (Hexadimethrine bromide)	Sigma	H9268	
Puromycin	Gibco	A11138-03	
RBC Lysis Buffer (10X)	Biolegend	420301	
Recombinant murine IL-2	Peprotech	212-12	Activation of isolated CD8+ T cells
Sodium pyruvate (100X)	Gibco	11360-070	
TruStain fcX (anti-mouse CD16/32) Antibody	Biolegend	101320	

: Nikon 10X objective (resolution 1.22 µm)
: Green channel excitation: 440 - 480 nm
: Green channel emission: 504 - 544 nm
: Red channel excitation: 565-605 nm
: Red channel emission: 625 - 705 nm

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article: REAL TIME DETECTION OF IN VITRO TUMOR CELL APOPTOSIS INDUCED BY ACTIVATED MOUSE CD8+ T CELLS TO EVALUATE IMMUNE SUPPRESSIVE FUNCTIONS OF TUMOR-INFILTRATING MYELOID CELLS

Author(s): T. KITAMURA, D DOUGHTY-SHENTON, T POLLARD, N CARRAGHER

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

jove
JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, Jo VE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	NEIL CARRASHER
Department:	CANCER RESEARCH UK EDINBURGH CENTER
Institution:	UNIVERSITY OF EDINBURGH
Title:	PROFESSOR OF DRUG DISCOVERY
Signature:	Neil Carr
Date:	28th JULY 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

This piece of the submission is being sent via mail.

This piece of the submission is being sent via mail.

This piece of the submission is being sent via mail.

Takanori Kitamura, PhD, DVM
Chancellor's Fellow
MRC Centre for Reproductive Health
The University of Edinburgh
The Queen's Medical Research Institute
47 Little France Crescent Room W1.04
Edinburgh EH16 4TJ
E-mail: tkitamur@exseed.ed.ac.uk
Phone: +44 (0)131 242 6764

RE: Revisions for our JoVE submission JoVE58841-[EMID:3957d6015f7f3e00]

30th October, 2018

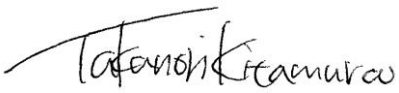
Dear Editor and reviewers,

Thank you so much for your time to review our manuscript. We have submitted the revision of our manuscript "Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells" by Kitamura et al.

We have addressed all comments from the editor and reviewers, and added new figures and extended explanations to the revised manuscript. Please see the following Point-by-point Response (original comments and our responses are written in black and blue respectively).

We hope this paper is now acceptable for publication in the “Journal of Visualized Experiments”

With best regards,



Takanori Kitamura

Point-by-point Response

Editorial comments:

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

→ We have proofread the article and corrected typos and grammatical errors.

2) Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

→ We have added this information in the first paragraph of the Protocol.

3) Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

1.2: Please mention how to confirm that cells are negative for mycoplasma.

1.3-1.5, 4.5: Please specify incubation temperature throughout the protocol.

2.1: Please specify the age and gender of mice. Are the mice anesthetized before injection?

2.3: What volume of PBS is used to wash? How many times?

2.5: Please provide composition of E-DMEM. Refer to Table of Materials if it is purchased.

2.6: How to count the number of live cells?

3.1: Please specify the euthanasia method and mention how to isolate the spleen.

3.2: What is used to grind?

3.5: What is used to adjust the cell concentration?

3.6: Please describe how this is actually done.

3.9: Please add a sub-step that describes the use of flow cytometry.

5.2.1: Please mention the reaction conditions for trypsinization.

5.2.2: How to adjust the density?

→ We have added sentences and information to address the above comments.

4) 7, 8 and their sub-steps: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

→ We used the commercial software specifically designed for this microscope system for the analysis, so we do not think that it would be reasonable to describe the analysis steps in such minute detail (i.e steps which refer to elements in the specific GUI for this software). Since, as stated in the manuscript we believe that the principles underlying the analysis can be adapted to other image analysis software, we have elaborated the manuscript to further describe the key parameters necessary for the image analysis which readers can adapt using their preferred software package (Sections 7 and 8). We have also added a link to the online Technical Note for the specific software we used in the Table of Materials, so that readers using the same software can use it as a resource.

5) After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the

essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

6) Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

7) Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

→ We have highlighted Steps 4-6 in the text, and attached a file showing the relevant sentences (Steps for Video).

8) Line 384: Please remove commercial language (Essen Bioscience).

→ We have removed the word "IncuCyte" and "Essen Bioscience".

9) Figure 2: Please make the scale bars more readable.

→ We have substituted a scale bar that is more readable. We have also changed the colors of arrowheads based on Reviewer's comments.

10) Figures 3-5, supplementary Figure 2: Please change "hr" to "h" for the time unit. Please include a space between the number and the unit of the scale bar in Figure 3.

→ We have changed this as suggested.

11) Please upload Figure 3 individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .pdf, .psd, or .ai file.

→ We have uploaded the files as requested.

12) Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

→ One of the critical steps is distinguishing apoptotic target cells from dying effector cells. In the protocol section, we have mentioned this point and listed controls that should be used (Step 5.7) as well as detailed parameters to set up the masks for accurate detection of apoptotic targets (Steps 7.1 – 8.4). We have also discussed limitations of the current protocol and potential solutions within the Discussion.

13) Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

→ We have added the lot numbers.

14) References: Please do not abbreviate journal titles.

→ We have changed this as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript presents a real-time monitoring method for T-cell mediated tumor cell lysis that can be used to measure the effect of immunosuppressive or immunostimulatory factors, such as immunosuppressive TAMs and MDSCs. The authors provide a very good assessment of existing methods and their shortcomings. This could be a powerful technique with broad applications. Overall, this manuscript has potential, but it needs to address some issues and undergo some revision.

→ Thank your for your careful reading and constructive comments.

Major Concerns:

Figure 2 and 3 are a bit confusing to interpret. These are the key figures that the entire paper depends on, but they are not convincing. The movies didn't do much to resolve this. Figure 2: It seems like there's quite a bit of variability in the red expression. It would be helpful to also show the red and green signals without the phase contrast overlay. The one cell that was identified as dead has so many effector cells over it that it is difficult to discern if the cas-3 is fluorescing in the target or effector cells. It's obscured by the size exclusion mask. Maybe zoom in on that better or pick a different field to show. It would be nice to include what the purple and teal masks and the filled arrow are in a figure legend, like the "excluded from the mask" arrow is. This would be extremely helpful for Figure 3 also. I also would like to see more fields shown. Why are so many more effector cells dying than target cells? Is that normal for these types of coculture experiments?

→We have changed Figure 2 to show a representative field with higher magnification. We have also added images without phase contrast and legends in the figure.

→ It is indeed normal and inevitable that more dead effector cells will be present than target cells in this type of co-culture assay. Since we added a 4-fold higher number of effector cells compared to target cells, green (apoptotic) effector cells were more frequently found in each field. However, this does not present a problem in detecting apoptotic cancer cells as we can exclude the smaller apoptotic effector cells from analysis by use of the size-restriction mask and detect only instances of red/green overlap as apoptotic nuclei of dying cancer cells.

Figure 3: What is going on with the small, rounded target cells in the second row. They look like they are dead or dying but are not fluorescing green. Is this a potential problem for data analysis? Also, the loss of the red signal in your apoptotic cell could complicate your colocalization analysis. It would be helpful if it were more clear that the arrows are showing the same cells in each timepoint. Pick colors other than blue and purple. Maybe yellow for the dead cells? Since we expect colocalization, it would be more intuitive of a color choice. Adding a legend for what the arrows mean would make interpretation of the figure more straightforward.

→ The small rounded target cells represent the cancer cells undergoing mitosis, which can be seen in the video. Existence of the mitotic cells does not affect data analysis, as these cells are not undergoing apoptosis (and thus are not showing green fluorescent signal). In order to make this point clear, we have shown the images as a supplementary figure and explained this in the text (second paragraph of Representative Results).

→ We have changed the colors of the arrowheads based on the suggestion. We have also modified a legend to explain that arrowheads are tracking the same cells through the different fields/time point.

There are entirely too many mis-numbered and mis-referenced sections, figures and figure legends. This became more frustrating as the paper went on. In many places, more details are warranted. It would be helpful to have more information about the most important reagents (Lenti and cas-3) within the text rather than only relying on the table.

→ We have addressed the issues of mis-numbering and referencing in the revised manuscript and have also elaborated upon many details throughout the manuscript. The journal guidelines do not permit us to name the commercial reagents or provide in-depth explanation of their details in the text. We have thus provided the sources and catalogue numbers in Table of Materials.

Minor Concerns:

1) Figure 4 and all the supplementary figures are duplicated.

→ This is caused by an unexpected system error. We have fixed the problem.

2) Figure 5: It would be more appropriate to consider the statistical analysis of the data in its entirety rather than pairwise comparisons at each timepoint. Also, what is the statistical test used? Instead of comparing to target only, would it be more appropriate to compare to the E + T to show that

you're actually getting statistically significant reduction in killing? The current results showed that statistically significant killing is occurring even with the suppressors.

→ Following your suggestion, we have changed the analysis to determine the area under the curve, where the curves depict the apoptotic fraction of the target cell population at each time point. We then used unpaired t-test with Welch's correction to determine statistical significance.

3) Supplementary Figure 1 Eliminate the contour plots. They don't add anything to the analysis. Your gating for MAMs is wrong. You're clearly including the lower portion of the MDSC population into your gate. Therefore, all of your MAM data is contaminated with MDSCs.

→ We have deleted the contour plots.

→ M-MDSCs and MAMs are distinguished as $CD11b^{high}Ly6C^{high}$ and $CD11b^{high}Ly6C^{low}$ respectively. The threshold of Ly6C level to distinguish MAMs and M-MDSCs is based on that of resident alveolar macrophages (RMAC). We have mentioned this point in the text (2.4). It has been reported that MAMs and M-MDSCs isolated by this gating strategy have different morphology, distinct gene expression profile, and different mechanisms to suppress $CD8^+$ T cell cytotoxicity (*Frontiers in Immunology* (2018) 8:2004).

4) Supplementary Figure 3: The lack of proliferation in the CD3/28 antibody stimulation condition is troubling.

→ We found a significant increase in the proliferation of T cells cultured with CD3/CD28 antibody, this was further increased by a co-culture with MAMs or MDSCs.

5) Reference the methods table somewhere early in the text so that we know it's here. You said you used 70 & 40□m cell strainers in the text but your table says 100 and 40□m.

→ We added the following sentence in the first paragraph of the Protocol; "Information about commercial reagents and equipment are listed in a supplementary Table of Materials."

→ We used 100 and 40 µm strainers, and corrected the typo in the text.

6) Some specific thoughts while reading include, labelled by line number:

51 How long is the cas-3 substrate detectable after a cell has undergone apoptosis?

→ It is difficult to determine exactly how long the caspase-3 signal lasts as the cells migrate across the plate bottom and some cells undergo mitosis during the experiment making it difficult to track individual cells without using much shorter timepoints than employed in these experiments. However, based on our time-lapse images, we estimate that green fluorescence from the caspase substrate was detectable for 15 – 20 h after first appearing in a cell undergoing apoptosis.

62 "Progress" seems like an odd word choice.

→ We changed the word "progress" to "initiate".

101 Clarification of "metastasis-associated macrophages" compared to tumor-associated macrophages would be helpful.

→ We have added a paragraph explaining MAMs and M-MDSCs (the last paragraph of Introduction).

106 Why are figures 1 & 2 duplicated?

→ This is simply an error in uploading the files. We have fixed the problem.

114 Would this approach be appropriate for human cells as well? It seems like it would, but addressing that would help potential adopters of this technique.

→ Technically, it is possible to apply our assay to investigate human cells, as activation antibodies against human CD3 and CD28 are commercially available, and a protocol to isolate human TAMs from clinical samples has been established. We have described it in Discussion.

125 Define "low". What is the target cell number or density at lentivirus addition?

→ We have changed the sentence as following; "If the proliferation rate of target cells is low (population doubling time greater than 36 h), the number of cells can be increased to $1x10^4$."

135 How long is necessary to incubate with puromycin at this dose. Also, that dose may not be effective for all cell types.

→ We have explained that one should "culture the cells until they are 80-90 % confluent". We also added the following note: "Concentration of puromycin will be different between target cell types, and should be optimised using un-transfected cells."

160 Could you include references to support your choice of markers, especially since you're using "MAMs" instead of "TAMs".

→ We included references that show gating strategy to detect MAMs (references 10, 11, and 14).

166 E-DMEM needs to be defined here as well.

→ We defined the contents of E-DMEM in steps 2.5 and 3.8.

213 This should be moved to 4.4. Why is CSF1 in T cell activation medium? They shouldn't express the receptor.

→ We moved it as suggested. CSF1 is not required for T cell activation, but is essential for survival of MAMs and M-MDSCs. We have mentioned this as a note for Step 4.3.

217-219 Number references are wrong.

→ Thank you for pointing out this error. We have corrected the typo.

238-252 Stick with scientific notation for cell numbers for consistency.

→ Based on the suggestion, we utilized scientific notation throughout the text.

245 I would like to see data on purity. Mouse TAMs release from the plastic with vigorous pipetting.

→ We cannot perform a purity check due to the small number of cells. Instead, we have attached some images demonstrating that mouse MAMs are not detached from the well after the collection of co-cultured T cells. We have also shown the representative images showing the morphology of T cells and MAMs cultured in an imaging plate. As shown in the figure, MAMs are obviously larger than T cells and show granules in their cytoplasm. Since we don't find such a cell type in our time-lapse images, the contamination of MAMs must be negligible.

264 Define "too many". What are approximate cell density limits?

→ Our preliminary experiments suggest that an initial effector to target ratio in 8:1 (i.e., total $9x10^3$ cells per well) presents difficulty in distinguishing apoptotic target cells from effector cells in our microscope system due to the increased likelihood of aggregates of apoptotic effector cells overlaying non-apoptotic target cells being falsely detected as co-localization. We have mentioned this point in the note of Step 5.7.

267 "Should" is too gentle. Include a list somewhere of all of the controls that are needed for proper data analysis.

→ We have modified this in the note for Step 5.7 as follows: "Wells containing the following four controls are necessary to aid with data analysis: target cells at the density used for the co-culture wells ($1x10^3$ cells/well) in medium with and without caspase-3 substrate, effector cells ($1x10^3$ cells/well) in medium with and without caspase-3 substrate."

284 What are the actual fluorophores used?

→ We used mKate2 for the nuclear-restricted red fluorescence and a fluorophore with excitation at 488nm for the green fluorogenic activated caspase-3 substrate. We have added this information in the text (Step 6.2), and listed the associated commercial reagents in the Table of Materials.

288 How long is the cas-3 detectable for after a cell undergoes apoptosis? Showing control data on that would be very helpful.

→ As mentioned above, it is difficult to determine exactly how long the caspase-3 signal lasts as it is difficult to track individual cells without using much shorter timepoints than those employed in these experiments. As this is a population-based experiment and we calculated the fraction of the target cell population that is apoptotic at any particular time point, this does not present a problem for analysis.

314 It sounds like you're including a significant portion of the effectors in your size exclusion mask. A figure with the nuclear sizes of the target and effectors with where to set the threshold would help others.

→ We have updated the methods (Section 7) to list the specific values we used with the specific software employed in these experiments. We have also updated Figure 2 to better depict how the size exclusion mask allows detection of apoptotic target cells.

320 Give more detail on this step and the data analysis in total. Also, your figures clearly show the loss of the red nuclear signal with the gaining of cas-3 signal, so you shouldn't actually get colocalization of signal. How does the analysis address this very significant issue?

→ As seen in Figure 2 there is a period of time during which there is co-localization of the 2 signals that is evident when the individual fluorescent channels are examined. We have updated Figure 2 to show the green and red fluorescent channels in isolation, which makes this co-localization clearer.

334 Is dividing or subtracting more appropriate here?

→ We have updated our analysis to express the data as the fraction of the population that is apoptotic at each time point. For this analysis procedure, dividing is appropriate particularly as there may be small differences in the number of cells in each well or image and expressing the apoptotic cells as a fraction of the population gives greater accuracy.

367 Figure 3 doesn't really represent that well for MDSCs.

→ Given this comment, we have selected another representative field and shown it with higher magnification, which shows that target cells are associated with T cells but do not undergo apoptosis.

391-2 The colors used for the masking aren't ideal.

→ We have changed the colors for the mask. We have also replaced the images with those of higher magnification, which we believe makes it clearer how each mask works to produce more accurate analysis.

Page 10 There are a bunch of mis-referenced figure numbers here and you're missing an entire legend.

→ We are sorry about the errors. We have scrutinized the manuscript and corrected the errors.

441 This seems counter to what other groups have shown. Do you have any other references to support this conclusion?

→ So far, there is no reference reporting the enhancement of $CD8^+$ T cell proliferation by macrophages. However, we have reproducibly found that MAMs enhance antibody-induced proliferation of $CD8^+$ T cells, whereas the expanded $CD8^+$ T cells exert less cytotoxicity. This is a novel finding that emphasizes the importance of the cytotoxicity assay to evaluate the immune suppressive function of macrophages.

453 Did you ever titrate the target cell numbers? That could possibly confirm or refute your conclusion.

→ This is a valid point. Since we have not titrated target cell numbers we have removed the line in the manuscript, which speculates on the effect of target cell mitosis on the level of apoptosis observed.

468-9 Can you quantify that and show it? That would be a huge power of this method that you're not really exploiting.

→ Technically it is possible to quantify the frequency and duration of effector-to-target interaction utilizing the basis of this assay system. However, it requires the acquisition of higher magnification images with shorter imaging intervals, which is not suitable for the scope of this protocol, i.e., to determine apoptotic fraction of target cell population. We intend to investigate this in future studies into the mechanism underlying suppression.

470 Only that many cells per well.

→ We added the word “per well” (the third paragraph in the Discussion).

486 I find it difficult to believe that your microscope signal doesn't have a DAPI channel.

→ The microscope that we use was specifically developed for live cell imaging particularly long-term live cell imaging (days or weeks) and the microscope resides in the cell culture incubator (please refer to Table of Materials for manufacturer and specific model). The manufacturer likely does not include a DAPI channel as the excitation for DAPI is in the ultraviolet range of the spectrum and repeated excitation over long-term imaging may cause DNA damage of the experimental cells leading to cell death.

516 What's the difference between this manuscript and reference 6?

→ In the reference 6, the apoptotic target cells are detected by flow cytometry or fluorescent microscopy at a fixed time point. In contrast, our assay enables acquisition of time-lapse images and thereby identification of the time of peak apoptosis. As we described in the first paragraph of the Discussion, this is one major advantage of this assay since determination of an optimal time point is important for appropriate comparisons between different conditions. Furthermore, analysis masks utilized in our protocol (target detection mask in red channel, size restricted apoptosis mask in green channel, and their combination, red/green overlap mask) enable more accurate detection of apoptotic target cells.

Reviewer #2:

Manuscript Summary:

In manuscript entitled "Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells" Takanori Kitamura and co-authors present in vitro multi-step assay for evaluation of: a) immunosuppressive function of murine MDSCs and TAMs; b) CD8 T cell cytolytic activity against tumor cells. Using real time fluorescence microscopy authors were able visualize the T cell-mediated apoptosis of tumor cells.

This is interesting novel approach to evaluate both immunosuppressive activity of myeloid cells and T cell anti-tumor activity using one multi-step assay. However, several concerns that should be clarified by authors.

Major Concerns:

1) Authors using CD3/CD28 Ab pre-activated CD8 T cells for generation of tumor cell cytolytic (CTL) activity. Since, no antigen has been used for generation of CTLs, authors should mention that this assay is antigen non-specific.

→ We have mentioned it in the fifth paragraph of the Discussion.

2) It is not clear, why authors cultured isolated tumor-infiltrating macrophages and m-MDSCs in the presence of recombinant M-CSF. Recombinant M-CSF is potent inductor of macrophage

differentiation, which drives differentiation of monocytic MDSCs toward macrophages. As a result, authors evaluated immunosuppressive activity of macrophages only (MAMs and MDSC-derived macrophages).

→ CSF-1 (M-CSF) is an essential growth factor for survival of classical monocytes and their derivatives including M-MDSCs and MAMs. We thus need to add recombinant CSF-1 in the culture to maintain the viability of the suppressor cells. The same medium was used for the T cell co-culture with target cancer cells to keep conditions consistent. CSF-1 is found in nano-molar concentrations in all tissues, and is required for monocyte/macrophage viability *in vivo* and thus this is a physiological context for these cells. We have mentioned this point in the note for Step 4.3.

→ Using this protocol, we have identified that cytotoxicity of $CD8^+$ T cells cultured with MAMs and M-MDSCs were suppressed via different mechanisms (i.e., CTLA4 and ROS mediated mechanism respectively). It is therefore likely that this assay can evaluate the immunosuppressive activity of both MAMs and their progenitor M-MDSCs.

3) It would be interesting to know whether MAMs (F4/80+/Ly6c-) or tumor-infiltrating m-MDSCs (Ly6c+) are able to kill syngeneic T cells via apoptosis?

→ As shown in Supplementary Figure 3, MAMs and MDSCs isolated from the metastatic lung of C57BL/6 mice promote, rather than suppress, the proliferation of $CD8^+$ T cells from syngeneic mice. Furthermore, we did not find a significant reduction in the ratio of live $CD8^+$ T cells cultured with MAMs or MDSCs compared to the cells in mono-culture. It is therefore unlikely that tumor-infiltrating myeloid cells induce apoptosis of T cells in our model.

Minor Concerns:

None

Figure for Reviewer#1. The contamination of MAMs in our time-lapse imaging should be negligible.

A) Representative images of wells in the first culture plate before (top row) or after (bottom row) collecting $CD8^+$ T cells. $CD8^+$ T cells were cultured for 4 days without (left column) or with (right column) MAMs, and were collected by gentle pipetting as described in the Protocol. After the collection of T cells, large attached cells (i.e., MAMs) were found in a well of co-culture, which were not found in a well of T cell mono-culture.

B) Representative images showing the morphology of $CD8^+$ T cells and MAMs cultured in an imaging plate. $CD8^+$ T cells and MAMs were isolated as described in the Protocol, and seeded into wells of the second culture (imaging) plate. After 18 h, images of the cultured cells were acquired. MAMs are obviously larger than T cells and show granules in their cytoplasm.