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Quantitative Evaluation of Tumor-specific T Cells in Tumors and Lymphoid Tissues

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Abstract

Homing of tumor-specific cytotoxic T lymphocytes (CTLs) to the tumor tissues represents a vital step in procuring an effective anti-tumor immune response. Intratumoral accumulation of tumor-specific CTLs can be supported through local chemokine modulation using immune adjuvants or viral vectors, as well as vaccination, using peptide, protein or cell-based vaccines, including dendritic cell (DC) vaccines. Clinical and pre-clinical studies demonstrate that the current immunotherapy regimens are only effective when high numbers of CTLs are present within the tumor microenvironment (TME). Notably, many types of cancer take advantage of this principle and restrict T cell migration into the tumor, subverting the anti-tumor immune response and allowing uncontrolled tumor growth. This chapter discusses the mechanisms involved in the migration of CTLs into tumors and describes the feasible method of evaluating treatment-induced changes in the numbers of polyclonal tumor-specific CTLs in the TME and lymphoid tissues. The described method is widely applicable to multiple tumor models with wild-type antigen expression patterns, without the need for genetically-manipulated cancer cells or animals expressing defined T cell receptors.

Keywords

cancer; cytotoxic T lymphocyte (CTL); dendritic cell (DC); ELISpot; immunotherapy; murine models; oncology; tumor associated lymphocyte (TAL); tumor infiltrating lymphocyte (TIL); tumor microenvironment (TME)

Introduction

The accumulation of cytotoxic T cells (CTL) in the tumor microenvironments (TME) is an important prognostic factor for cancer patients and one of the key factors predicting the effectiveness of different forms of immunotherapy. Studies in multiple patient cohorts demonstrated that TME infiltration by CTL is uniformly predictive of prolonged survival

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(Fridman et al., 2011) in several cancer types including colorectal (Pages et al., 2005; Galon et al., 2007), ovarian (Zhang et al., 2003; Sato et al., 2005), bladder (Sharma et al., 2007), breast (Mahmoud et al., 2011; X. Li et al., 2019), melanoma (Azimi et al., 2012; Rahbar et al., 2015), pancreatic (Carstens et al., 2017). In contrast to the prognostically favorable CTLs (Pages et al., 2005; Galon et al., 2006; Anraku et al., 2008; Yamada et al., 2010), intratumoral regulatory T cells (T_{regs}) predict poor outcomes (Curiel et al., 2004; Clarke et al., 2006; Michel et al., 2008; Chaput et al., 2009), indicating therapeutic potential for selectively enhancing intratumoral CTL densities, relative to T_{regs} , or converting “cold” tumors into “hot” ones.

The accumulation of suppressive immune cells including T_{regs} (Shang et al., 2015), myeloid derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) are important to consider when evaluating the TME. These subsets of suppressive cells produce such suppressive factors as IL-10, TGF β , IDO, arginase and nitric oxide and can directly and indirectly inhibit the efficacy of ICI (Shang et al., 2015; Binnewies et al., 2018; Cassetta & Kitamura, 2018; Weber et al., 2018). In a clinical setting, frequency of circulating MDSCs is associated with a poor response to the ICI α CTLA-4 (Meyer et al., 2014; Weide et al., 2014; Martens et al., 2016; Sade-Feldman et al., 2016) and thus neutralization of this subset of cells has become an important topic of ongoing research (Weber et al., 2018). Further, suppressive cells can express the ligands for ICI including PD-L1/2 (PD-1) and CD80/86 (CTLA-4) (Kryczek et al., 2006; Kuang et al., 2009; Bloch et al., 2013).

The presence of tumor-specific T cells in the TME is particularly important for the ability of the individual patients to respond to immunotherapy with immune checkpoint inhibitors (ICI), such as the inhibitors of the programmed cell death-1 (PD-1) pathway, as demonstrated by the correlations between CTL accumulation and clinical outcomes of ICI (Ji et al., 2012; Herbst et al., 2014; Tumeh et al., 2014; Llosa et al., 2015). The presence of these cells, including tumor-infiltrating lymphocytes (TIL), immune cells that have exited the bloodstream and entered a solid tumor, and tumor associated lymphocytes (TAL), immune cells present within the intraperitoneal ascites of cancers including ovarian cancer, have the ability to combat the growing cancer once in proximity of the tumor (Matsuzaki et al., 2010; Huang et al., 2017). However, the systemic numbers of tumor-specific T cells in circulation are very poor predictors of clinical responses (Astsaturov et al., 2003; S. A. Rosenberg et al., 2005). This striking distinction highlights the key importance of CTL migration to the tumor site, in addition to their induction in the effectiveness of anticancer immunity. Thus, methods which promote CTL accumulation in or around the tumor have the potential to expand the benefit of ICI and potentially other cancer treatments to very large groups of the currently non-responsive patients.

While ICI is becoming the gold standard of care for many cancers, their effectiveness differs between different patient groups. While the response rates in melanoma (Brahmer et al., 2012; Hamid et al., 2013; Topalian et al., 2014; Luke & Ott, 2015), non-small cell lung cancer (Reck et al., 2016; Kanwal et al., 2018), bladder cancer (J. E. Rosenberg et al., 2016; Sharma et al., 2016; Bellmunt et al., 2017; Rouanne et al., 2018) and renal cell cancer (Topalian et al., 2012; Motzer et al., 2015; Weinstock & McDermott, 2015) can reach 40%,

the overall response rates remain around the 15 – 20% (Xu-Monette et al., 2017; Ribas & Wolchok, 2018).

To enhance the percentage of the patients with clinical benefit, multiple combination therapies have been pursued (Atkins & Tannir, 2018; Johnson & Win, 2018; Tang et al., 2018), aiming to make “cold” tumors become “hot” (Trujillo et al., 2018). Three main types of interventions tested in this regard include activation of endogenous antigen presenting cells (APCs), direct intratumoral delivery of CTL-attracting chemokines and the modulation of the TME using different chemokine inducers and modulators.

Local activation of the stimulator of interferon genes (STING) pathway within the TME was shown to be an effective way of local DC activation and type-1 IFN production in mice, leading to T cell mediated anti-tumor immunity (Fuentes et al., 2013; Ohkuri et al., 2014; Woo et al., 2014). Alternative strategies include the use of vaccines which target exogenous antigens to DCs (Kreutz et al., 2013), ex vivo generated DC vaccines, such as Sipuleucel- T (Provenge®) (Kantoff et al., 2010; Fong et al., 2014), genetically-modified viruses, such as talimogene laherparepve (B. L. Liu et al., 2003; Ribas et al., 2017), or the delivery of chemokine genes such as CXCL11 (Francis et al., 2016; Z. Liu et al., 2016). Our alternative strategy, already being tested in early phase clinical trials include combinatorial chemokine modulating (CKM) regimen which can induce local intratumoral effect after either systemic or local administration (Obermayer et al., 2018) due to the enhanced susceptibility of the TME, compared to the surrounding healthy tissues (Muthuswamy et al., 2012). Examples of effective CKM combinations include type I IFNs combined with TLR ligands (Lu, 2014; Muthuswamy et al., 2015), IL-18 (Wong, Berk, et al., 2013; Wong, Muthuswamy, et al., 2013) and COX2 inhibitors (Wong et al., 2016; Theodoraki et al., 2018).

In order to facilitate preclinical evaluation of new therapeutic agents and their combinations effective in promoting intratumoral accumulation of tumor-specific CTLs, we developed a simple and generally applicable protocol to evaluate the numbers of tumor-specific CTLs in the TME, without the need for genetically-manipulated cells or animals, and cell tracers (Figure 1). In contrast to the existing alternatives, which use T cells from genetically manipulated mice with defined T cell receptors (such as OT-I), and genetically modified tumor cell lines which uniformly express highly immunogenic model antigens, such as ovalbumin (OVA) (reviewed in (Dranoff, 2011)), our model evaluates the numbers of all T cells able to recognize tumor cells (rather than their selected antigens) and produce IFN γ in result of such recognition.

The ability to evaluate polyclonal T cell responses to multiple tumor-related weak antigens (mutated or over-expressed “self” antigens) eliminates the need for surrogate antigens or genetically-manipulated T cells with artificial TCRs, and allowing the evaluation of T cells recognizing different TAAs expressed by cancer cells. It eliminates the need for known cancer antigens and arbitrarily selected threshold of antigen specificity, reflected in the design of tetramers or defined TCR α/β combinations. Our protocol allows for enumeration of spontaneously-arising and immunotherapy-induced T cells which are able to recognize any cancer cell, without the need for prior identification of the relevant immunogenic epitopes. This eliminates any bias resulting from the selective evaluation of high affinity

TCR-antigen interactions, and assures its immediate general applicability to multiple tumor models and mouse strains. We have tested this protocol with various murine tumor models including TIL extraction from MC38 tumors and TAL extraction from ID8 tumors (Obermajer et al., 2018), but anticipate that it can be used in multiple other tumor models.

Materials

Reagents

1. AN18 anti-IFN γ -coating antibody (Mabtech)
2. Phosphate buffered saline (PBS) (Gibco)
3. Ethanol 70% (vol/vol) diluted (EMD Serono)
4. RPMI-1640
5. Cell culture medium (CCM) should be prepared ahead of TME. Supplement RPMI-1640 (Gibco) medium with:
 - a. 1% (vol/vol) 100 U/ml penicillin-streptomycin (Gibco)
 - b. 1% (vol/vol) 1 mM sodium pyruvate (Invitrogen)
 - c. 1% (vol/vol) non-essential amino acids (NEAAs) (Gibco)
 - d. 0.1% (vol/vol) 14.3 mM 2 β ME (Sigma-Aldrich)
 - e. 1% (vol/vol) 2 mM L-glutamine (Gibco)
 - f. 10% (vol/vol) FBS
6. Digestion buffer is freshly prepared with reconstituted (according to manufacturer instructions) Mouse Tumor Dissociation Kit (Miltenyi) enzymes in the following relative amounts (can be scaled in 2.5 ml increments according to experiment size):
 - a. 100 μ l enzyme D
 - b. 50 μ l enzyme R
 - c. 12.5 μ l enzyme A
 - d. 2337.5 μ l CCM
7. ACK Lysis buffer (Life Technologies)
8. Trypan blue (0.4% (wt/vol)) solution (Corning Cellgro)
9. 10x acidic (pH: 4.6. 1.051 g/ml) PBS is prepared with:
 - a. 13.5 g NaCl
 - b. 0.125 g Na₂HPO₄·2H₂O
 - c. 2.1 g KH₂PO₄
 - d. 200 ml dH₂O.

10. Standard Isotonic Percoll Solution (SIP) is freshly prepared by mixing Percoll with acidic PBS (see point 9 above) at 10:1 ratio.
11. 60% (vol/vol), 45% (vol/vol) and 34% (vol/vol) SIP solutions are prepared for separation steps by diluting SIP with CCM
12. Fetal bovine serum (FBS) (Gibco) is stored in bottles direct from manufacturer at -20°C . Bottles are thawed slowly at 4°C and then heat inactivated for 30 min at 56°C . Heat-inactivated aliquots are stored at -20°C
13. Ethylenediaminetetraacetic acid (EDTA) (Life Technologies)
14. Wash buffer is made from PBS with 0.05% (vol/vol) Tween 20 (Fisher Scientific)
15. Bovine serum albumin (BSA) (Sigma-Aldrich)
16. R4-6A2 anti-IFN γ biotinylated detecting antibody (Mabtech) diluted in PBS/ 0.5% (wt/vol) BSA
17. ABC Reagent (Vector Laboratories)
18. ELISpot AEC substrate kit (Vector Laboratories)
19. dH $_2$ O (Life Technologies)

Mice/splenocytes

1. Tumor cells matching the cells used to inoculate the experimental mice are used as target cells in the ELISPOT assay
2. Mice bearing specific tumors are to be treated according to IACUC approved protocols

Equipment/Supplies (with examples of our current suppliers)

1. 10, 200, 1000 μl Pipette tips (Eppendorf)
2. 20 – 200 μl multichannel pipette (Eppendorf)
3. P10, P200, and P1000 Pipetman (Eppendorf)
4. 2, 5, 10 and 25 ml sterile, individually wrapped Pipettes (Falcon)
5. Pipet-Aid (Corning)
6. ELISpot plates (Millipore)
7. Parafilm (Bemis)
8. Scissors (Fisher Scientific)
9. Forceps (Fisher Scientific)
10. Scalpel (Bard-Parker)
11. Cell culture incubator set to 37°C and 5% (vol/vol) CO $_2$ (Fisher Scientific)
12. 70 and 100 μm cell strainers (Falcon)

13. 3 ml syringe with the removable piston (Covidien or analogous)
14. 15 and 50 ml polypropylene conical tubes
15. Centrifuge
16. Microscope
17. Hemocytometer
18. Gamma irradiator
19. ELISpot reader

Methods

Preparation I

1. Monitor experimental mice and continue to Step 2 when they are nearing experimental endpoint based on pre-determined IACUC approved parameters
2. Add 100 μ l of 15 μ g/ml AN18 and α IFN- γ coating antibody in PBS to each well of an ELISpot plate
3. Wrap the plate in parafilm and incubate overnight at 4°C
4. At experimental endpoint, euthanize mice in accordance to institutional guidelines

Tumor infiltrating lymphocytes (TIL)/Tumor associated lymphocyte (TAL) Isolation

5. To harvest solid (i.e. transplantable subcutaneous, autochthonous, spontaneous) tumors and isolate TALs from each individual mouse:
 - a. Sterilize the abdomen area of the mouse with 70% ethanol
 - b. Using scissors make a small incision into the peritoneal cavity
 - c. Using forceps and/or scissors collect the tumor tissue directly into a 15 ml conical tubes filled with 2 ml PBS
 - d. Add ~2 ml of PBS onto a petri dish (cover the bottom) and transfer the tumor tissue
 - e. Using two scalpels, mince the tissue to pieces <0.5 mm
 - f. Transfer the tumor pieces to a new 15 ml tube and add ~4 ml of digestion buffer (cover the tumor pieces)
 - g. Incubate tumors at 37°C for 30 min while rocking
 - h. Wet a 100 μ m strainer placed on top of 50 ml tube, transfer the tumor pieces onto the strainer
 - i. Mash the tumor pieces with the piston of a 3 ml syringe
 - j. Continuously wash with PBS to a final volume of ~20 ml

6. To harvest tumor ascites (i.e. transplantable intraperitoneal) and isolate TAL from each individual mouse:
 - a. Sterilize the abdomen area of the mouse with 70% ethanol
 - b. Fill a 10 ml syringe with 5 ml PBS and use a 18G needle to inject the PBS into the lower left quadrant of the abdominal
 - c. Aspirate as much peritoneal fluid as possible being careful not to penetrate organs within the cavity
 - d. Transfer the peritoneal fluid into 50 ml conical tube

Preparation of TIL/TAL for accessing T cell activity

11. Centrifuge the TIL (Step 5) or TAL (Step 6) at 400 g for 3 – 5 min at room temperature
 - a. Discard the supernatant and resuspend the cells in 1 ml ACK Lysis buffer to remove red blood cells
 - b. Add an additional 3 ml of ACK Lysis Buffer on top and invert tube to mix
 - c. Incubate the cells at room temperature for 4 min
 - d. Add 30 ml of PBS and centrifuge the cells at 400 g for 3 min at room temperature
 - e. Resuspend in 20 ml PBS and transfer over 70 µm strainer
 - f. Count the cells using hemacytometer
 - g. Centrifuge the cells at 400 g for 3 – 5 min at room temperature
 - h. Resuspend the cells in 3 ml SIP (see “Reagents”; points 9–10) and transfer to a 15 ml tube
12. Slowly add 3 ml of 45% (vol/vol) SIP on top of the 60% (vol/vol) SIP layer by expelling the 45% (vol/vol) SIP at a shallow angle down the side of the tube
13. Slowly add 3 ml of 34% (vol/vol) SIP on top of the 45% (vol/vol) SIP layer by expelling the 34% (vol/vol) SIP at a shallow angle down the side of the tube
14. Centrifuge the tubes at 2,400g for 30 min at room temperature without applying the brake
15. Collect the bottom interface (~2–3 ml) into a new 15 ml conical tube and fill the tube with PBS
16. Centrifuge at 600g for 10 min at room temperature
17. Resuspend the cell pellet in 500 µl CCM
18. Adjust volume of TIL/TAL to 1×10^6 /ml with CCM

Splenocyte Isolation

19. Harvest spleen and isolate splenocytes from each individual mouse
 - a. Sterilize the upper abdomen/chest area of the mouse with 70% ethanol
 - b. Using scissors make a small incision into the left flank of the mouse
 - c. Using forceps and/or scissors collect the spleen
 - d. Wet a 100 μ m strainer placed on top of 50 ml tube, transfer the spleen directly onto the strainer
 - e. Fill a 10 ml syringe with PBS and using a 22G needle inject into the spleen
 - f. Repeat 3 – 5x to thoroughly flush the cells from the spleen
 - g. Mash the spleen with the piston of a 3 ml syringe
 - h. Continuously wash with PBS to a final volume of ~20 ml
 - i. Centrifuge the cells at 400 g for 3 – 5 min at room temperature
 - j. Discard the supernatant and resuspend the cells in 1 ml ACK Lysis buffer to remove red blood cells
 - k. Discard the supernatant and resuspend the cells in 1 ml ACK Lysis buffer to remove red blood cells
 - l. Add an additional 3 ml of ACK Lysis Buffer on top and invert tube to mix
 - m. Incubate the cells at room temperature for 4 min
 - n. Add 30 ml of PBS and centrifuge the cells at 400 g for 3 min at room temperature
 - o. Resuspend in 20 ml PBS and transfer over 70 μ m strainer
 - p. Count the cells using hemacytometer
20. Resuspend splenocytes at 5×10^6 /ml in CCM

Preparation II

21. Wash the ELISpot plate (step 3) 3 TMEs with 200 μ l RPMI-1640 for 5 minutes
22. Block plate with 200 μ l CCM for 30 minutes at 37°C
23. Remove all CCM
24. Add 100 μ l of 1×10^6 TIL/TAL (step 12; total 1×10^5) to 4 wells of the ELISpot plate (see Figure 1 for recommended plate setup)
25. Add 100 μ l of 5×10^5 splenocytes (step 14; total 5×10^4) to 4 wells of the ELISpot plate

Preparation of cultured cancer cells as targets for assessing tumor-specificity of T cells

26. Harvest the relevant cancer cells from cultures, using 1 mM EDTA in PBS and centrifuge at room temperature
27. Resuspend cancer cells at 8×10^5 cells/ml CCM in a 50 ml conical tube
28. Irradiate with 20 Gy
29. To wash cancer cells, centrifuge at 400 g for 5 min at room temperature, resuspend in 20 ml PBS X2
30. Resuspend the cells at 4×10^5 cells/ml in CCM
31. Add 4×10^4 cancer cells in 100 μ l of to each of 3 wells with TIL/TAL and/or 3 wells with splenocytes (triplicate co-cultures with the cancer cell targets)
32. Add 100 μ l CCM to the remaining wells (TIL/TAL and/or splenocytes) for controls (triplicate control cultures).

ELISpot Procedure

33. Incubate plates at 37°C, 5% (vol/vol) CO₂ for 24 – 48 hours
34. Add 200 – 300 μ l of wash buffer to each well for 5 minutes $\times$ 5
35. Add 100 μ l of 1 μ g/ml of diluted R4–6A2 biotinylated secondary antibody
36. Incubate at room temperature for 2 hours
37. About 10 min before the incubation is finished, prepare ABC reagent according to the manufacturer's instructions
38. Add 200 – 300 μ l of wash buffer to each well for 5 minutes $\times$ 5
39. Add 100 μ l of ABC reagent, and incubate the plate at room temperature for 1 hour
40. Add 200 – 300 μ l of wash buffer to each well for 5 minutes $\times$ 3
41. Add 200 – 300 μ l of PBS to each well for 5 minutes $\times$ 2
42. While washing the plate, prepare AEC substrate according to the manufacturer's instructions
43. Add 100 μ l of AEC reagent per well.
44. Once spots are clearly visible (~5 – 20 minutes depending on assay) but before background appears, rinse the plate thoroughly with dH₂O
45. Remove the plastic lid from the plate and allow the plate to dry completely.
46. Read the plate using an ELISpot reader according to manufacturer instructions

Notes

Based on our experiences with this protocol we would like to draw your attention to some critical issues to pay attention to during experimentation.

1. We anticipate this procedure to accurately evaluate IFN- γ producing T cells in the tumor site and lymphoid organs in mice with any type of tumor. Our methods have been optimized for the isolation of immune cells from both solid tumors and tumor ascites. Although, we have successfully used this approach to evaluate multiple subcutaneous and intraperitoneal tumors including MC38 and ID8 (Obermayer et al., 2018), we cannot eliminate the possibility that some tumors may require additional methods to prepare single cell suspension of tumor cells and isolate T cells.
2. We recommend harvesting the tumors well in advance of the animals succumbing to high tumor burden, in order to minimize the impact of secondary effects of high tumor burden (tumor ulceration, infection, metabolic changes) which may complicate the interpretation of data.
3. We recommend isolating the T cells from the largest amount of tumor tissue as possible, to account for intratumoral heterogeneity of T cell infiltrates.
4. While dissociating the tumor, if tumor pieces are very small they can be directly transferred to a 2 ml Eppendorf tube with ~500 μ l digestion buffer. The entire contents of the tube can be transferred to the strainer.
5. An overnight rest period for all ex vivo cells used in this procedure including TIL, TAL, and splenocyte (Steps 14 and 16) is recommended.
6. Unrelated cultured cancer cells can be used as additional non-specific targets.
7. Depending on your model system, it may be advantageous to further enrich your splenocyte samples (Step 16) for CD4⁺ or CD8⁺ T cells using cell sorting or magnetic separation.
8. We have optimized the immune cell harvesting portion of this protocol for isolating total TILs or TALs from tumor-bearing mice. However, our protocol can be adapted to isolate other cell subsets by adding additional separating steps following Step 5/6. For example, to isolate T_{regs} additional steps to separate FoxP3⁺ by magnetic separation or flow cytometry based cell sorting methods could be included.
9. Although the production of IFN- γ is known to be well correlated with CTL activity, our protocol allows inclusion of additional antibodies in Step 2, to evaluate alternative markers, such as TNF- α or Granzyme B.

Discussion: applications of the Protocol

This protocol is designed to enhance the numbers of tumor-specific CTLs within tumor lesions (and lymphoid tissues), and to evaluate their changing numbers in the course of immunotherapy. A wide range of examples is provided in our recent publication (Obermayer et al., 2018) and include DC vaccines and different forms of systemically- or locally applied modulation of TME, such as systemic or local application of combinatorial adjuvants, intratumoral injection of DCs or different viral therapies.

We anticipate that the presented protocol may be used to identify the effective combinations of immune activators with chemo- or radiotherapy, blockade of tumor survival pathways (Vanneman & Dranoff, 2012), oncolytic virus therapies (J. Li et al., 2011), TNF/TNF-R superfamily ligands (Jensen et al., 2010), and/or blockers of inhibitory factors in tumor microenvironment (e.g. ICI, IL-10, M-CSF, VEGF, IDO- or COX2 blockers), to prioritize the most promising approaches for extended mouse- and eventual clinical testing.

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Figure 1: Key elements of the protocol.

Depending on the specific question, tumor-isolated TILs, or spleen-isolated cells, which may be further separated as CD4⁺ or CD8⁺ T cells, are rested overnights before testing of their specificity against the original cancer cells used in the tumor model of interest. The ELISpot plate(s) are processed after 24 hour incubation/co-culture. Placing sample from each mouse into 1 single column will easily organize your ELISpot plate for up to 12 experimental mice. TIL/TALs (Step 14) and splenocytes (Step 16) can be used across rows of the plate. Wells in which irradiated tumor cells are absent (Step 26) will serve as controls for each type of cell examined.