gibco

24 days of stem cells

Shape the future of stem cell innovation

October 1- November 1, 2019

Join us for 24 Days of Stem Cells; a premiere virtual event featuring the latest advances in stem cell research.

This year's format will feature a new hour of cutting edge content every week day starting October 1st. Attend the sessions that are most relevant to your work - at your convenience and at your pace.

During the 24-day long event, you can:

- Access leading scientific presentations from thought leaders around the world
- Watch live training demonstrations from our stem cell experts
- Download key stem cell tools and resources
- Complete weekly challenges to earn points towards certification and prizes

Register today at
www.24daysofstemcells.com

ThermoFisher SCIENTIFIC

WILEY

Mouse Model for Pre-Clinical Study of Human Cancer Immunotherapy

Zhiya Ya,[1] Yared Hailemichael,[2] Willem Overwijk,[2] and Nicholas P. Restifo[1]

[1]National Cancer Institute, Surgery Branch, Bethesda, Maryland
[2]Department of Melanoma Medical Oncology–Research, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas

This unit describes protocols for developing tumors in mice, including subcutaneous growth, pulmonary metastases of B16 melanoma, and spontaneous melanoma in B-Raf V600E/PTEN deletion transgenic mouse models. Two immunization methods to prevent B16 tumor growth are described using B16.GM-CSF and recombinant vaccinia virus. A therapeutic approach is also included that uses adoptive transfer of tumor antigen–specific T cells. Methods including CTL induction, isolation, testing, and genetic modification of mouse T cells for adoptive transfer by using retrovirus-expressing genes of interest are provided. Additional sections, including growing B16 melanoma, enumerating pulmonary metastases, tumor imaging technique, and use of recombinant viruses for vaccination, are discussed together with safety concerns. © 2015 by John Wiley & Sons, Inc.

Keywords: B16 melanoma • translational cancer immunotherapy • tumor imaging • use of recombinant viruses for vaccination

How to cite this article:
Ya, Z., Hailemichael, Y., Overwijk, W., and Restifo, N. 2015. Mouse Model for Pre-Clinical Study of Human Cancer Immunotherapy. *Curr. Protoc. Immunol.* 108:20.1.1-20.1.43. doi: 10.1002/0471142735.im2001s108

INTRODUCTION

In the past two decades, cancer immunotherapies have been beneficial in a subset of patients with metastatic melanoma or renal cell carcinoma, successfully treated by the administration of recombinant interleukin-2 (rIL-2), sometimes given together with autologous melanoma-reactive lymphocytes that have been expanded ex vivo (Rosenberg, 1999; Dudley and Rosenberg, 2003; Rosenberg et al., 2008). Recently, a number of different laboratories have used immune-modulating antibodies [genetically engineered autologous T cells expressing tumor-antigen-specific T cell receptor (TCR) or chimeric antigen receptor (CAR)] to treat a variety of human cancers other than melanoma and renal cell carcinoma. The list of cancer immunotherapies are now expanding to non-small-cell lung carcinoma (Brahmer et al., 2012; Topalian et al., 2012), metastatic hormone-refractory prostate cancer (Small et al., 2007), synovial sarcoma (Robbins et al., 2011), lymphoma, leukemia, breast cancer, colorectal cancer, ovarian cancer, and glioblastoma.

Animal models play important roles in translational cancer immunotherapy. They not only provide scientific principles for clinical trials, but can also be used to develop immune reagents such as humanized monoclonal antibody and HLA restricted antigen–specific TCRs that are directly applicable to cancer treatment. In addition, animal models allow pre-clinical studies on potential toxicity of therapeutic reagent in patients. Table 20.1.1 summarizes the pre-clinical studies in mouse models that have been translated into clinical trials in Surgery Branch, NCI, from 1986 to 2013.

Current Protocols in Immunology 20.1.1-20.1.43, February 2015
Published online February 2015 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/0471142735.im2001s108

Table 20.1.1 Summary of Pre-Clinical Mouse Models that were Translated into Clinical Trials of Cancer Immunotherapy in Surgery Branch, National Cancer Institute from 1986-2013

Cancer immunotherapy	Mouse model	Clinical trial
Tumor specific-vaccines		
Peptide anchor-modification	Irvine et al. (1999); Yu et al. (2004)	Rosenberg et al. (1998); Schwartzentruber et al. (2011)
Recombinant poxvirus	Irvine et al. (1997); Overwijk et al. (1999b)	Rosenberg et al. (2003); Lindsey et al. (2006)
Antibody immunotherapy		
CTLA-4	van Elsas et al. (2001)	Phan et al. (2003)
T cell immunotherapy		
TIL	Rosenberg et al. (1986)	Dudley et al. (2002)
Gp100 $_{209\text{-}217}$-specific T cells	Yu et al. (2004)	Morgan et al. (2006)
Gp100 $_{154\text{-}162}$-specific T cells	Johnson et al. (2009)	Johnson et al. (2009)
CEA $_{691\text{-}699}$-specific T cells	Parkhurst et al. (2009)	Parkhurst et al. (2011)
MAGE A3$_{112\text{-}120}$-specific T cells	Chinnasamy et al. (2011)	Morgan et al. (2013)
Less differentiated T cells	Hinrichs et al. (2009)	Dudley et al. (2010)
CAR immunotherapy		
Anti-VEGFR-2 CAR	Chinnasamy et al. (2010)	Phan and Rosenberg (2013)
Anti-CD19 CAR	Kochenderfer et al. (2010)	Kochenderfer et al. (2013)
Immune modulation		
Total body irradiation	Wrzesinski et al. (2010)	Dudley et al. (2008)
IL-12	Kerkar et al. (2010)	Manuscript in preparation
IL-15	Klebanoff et al. (2004)	Conlon et al. (2014)

This unit will detail protocols for developing tumors in mice including subcutaneous growth (Basic Protocol 1), pulmonary metastases of B16 melanoma (Basic Protocol 2), and spontaneous melanoma in B-Raf V600E/PTEN deletion transgenic mouse models (Basic Protocol 3). Two immunization methods to prevent B16 tumor growth are introduced in Basic Protocol 4 (using B16.GM-CSF) and 5 (using recombinant vaccinia virus). Therapeutic approach is focused on adoptive transfer of tumor-antigen-specific T cells (Basic Protocol 6). Identifying human HLA-restricted T cell receptors is described in Basic Protocol 7. In support protocols, methods including CTL induction, isolation, testing, and genetic modification of mouse T cells for adoptive transfer by using retrovirus expressing genes of interest are provided. Additional sections, including growing B16 melanoma, enumerating pulmonary metastases, tumor imaging technique, and use of recombinant viruses for vaccination, are discussed together with safety concerns (Table 20.1.2).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

Table 20.1.2 Summary of Protocols in this Unit

Basic Protocols	Support Protocols
Tumor models	
Subcutaneous B16 (Basic Protocol 1)	Growing B16 tumor cells (Support Protocol 1)
Pulmonary metastatic B16 (Basic Protocol 2)	Enumerating pulmonary metastases (Support Protocol 2)
Spontaneous melanoma in transgenic mice (Basic Protocol 3)	Luciferase imaging (Support Protocol 3)
Tumor protection	
B16.GM-CSF vaccine (Basic Protocol 4)	Immunization with recombinant rVV, rFPV, or rAd (Support Protocol 4)
rVV TRP-1 immunization (Basic Protocol 5)	Detection of antibodies against MDA by ELISA (Support Protocol 5)
Therapeutic approach	
Adoptive transfer of T cells (Basic Protocol 6)	CTL induction (Support Protocol 6)
Development of HLA restricted antigen-specific T cells for clinical use (Basic Protocol 7)	CTL isolation and testing (Support Protocol 7)
	CTL maintenance (Support Protocol 8)
	Culture of Pmel-1 T cells (Support Protocol 9)
	Retroviral transduction of mouse T cells (Support Protocol 10)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

MOUSE MODEL OF SUBCUTANEOUS TUMOR

BASIC PROTOCOL 1

The subcutaneous model is widely used for the evaluation of therapy in many tumor models, including B16 melanoma. Upon subcutaneous injection into syngeneic C57BL/6 mice, B16 will form a palpable tumor in 5 to 10 days and grow to a 1-cm × 1-cm tumor in 14 to 21 days. When allowed to grow larger, the tumors often become necrotic in the center and begin to ulcerate or bleed; it is advisable to sacrifice the mice before this point. The typical dose used is 1–5 × 10^5 cells/mouse. For any new tumor type, test its growth with 1 × 10^4 to 5 × 10^6 cells/mouse to establish a suitable model. It is important to note that, for subcutaneous tumor growth experiments, a consistent injection technique is extremely important. Each mouse should show a clearly visible, defined "bleb" upon injection; if not, a new mouse should be used. Mice without a clear "bleb" will show delayed tumor growth or no growth at all.

Materials

B16 culture, 50% to 80% confluent (see Support Protocol 1)
Phosphate buffered saline (PBS, Life Technologies) or Hanks' balanced salt solution (HBSS, Life Technologies)
6- to 12-week-old female C57BL/6 mice
70% isopropyl alcohol prep (Webcol, Kendall Healthcare)

50-ml conical centrifuge tubes
Refrigerated centrifuge, e.g., Sorvall RC4
100-μm disposable cell strainer (Falcon)

Hair clipper (Wahl, Model 8761)
1-ml disposable syringes and 27 G, ½-in. needles
Calipers

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997a, and determining viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b), restraint of mice (*UNIT 1.3*; Donovan and Brown, 2006a), ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b), and subcutaneous injection of mice (*UNIT 1.6*; Donovan and Brown, 2006c)

Prepare B16 cells

1. Ensure that B16 cells (Support Protocol 1) are in the logarithmic growth phase when harvesting for injection about 50% to 80% confluent.

   Nondividing tumor cells from confluent flasks may not be as efficient in producing tumors.

2. Aspirate medium, rinse flask briefly with 3 ml TrypLE Express, and aspirate again.

   Rinsing helps remove fetal bovine serum (FBS), which otherwise dilutes the trypsin and inhibits proteolysis.

3. Add 5 ml TrypLE Express and tilt flask to ensure that all cells are covered. Periodically and firmly tap side of flask until cells detach and slide down the culturing surface.

   Do not leave cells in trypsin any longer than necessary, to ensure high viability.

4. Add 5 ml cold DMEM medium and pipet vigorously to obtain a single-cell suspension.

5. Transfer to 50-ml conical centrifuge tube and add 40 ml cold DMEM medium to neutralize trypsin.

6. Centrifuge the cells for 5 min at 663 × *g* (1500 rpm in Sorvall RC4 centrifuge), 4°C.

7. Decant supernatant and resuspend cells in ice-cold PBS or HBSS, aiming for 1–5 × 10^6 cells/ml.

8. Pass suspension through disposable cell strainer to remove any clumps. Count live cells using trypan blue (*APPENDIX 3A & 3B*; Strober, 1997a,b).

   Viability should be >90%.

9. Adjust cell concentration to 1–5 ×10^6 cells/ml in ice-cold PBS or HBSS.

Inoculate mice

10. Relocate work area to facility where 6- to 12-week old female C57BL/6 mice are kept. Maintain cells on ice.

    Inject cells as quickly as possible after preparation; viability slowly decreases over time, even on ice.

11. Hold mouse with one hand, shave abdominal hair with hair clipper, and sanitize the skin with a 70% ethanol prep.

    Handling and restraint of mice is described in Donovan and Brown (2006a). Mice can also be shaved in advance, especially when more than 50 mice are required in an experiment. This can help prevent tumor cells sitting on ice for a long period of time. Sanitizing the skin needs to be performed immediately prior to injection.

12. Resuspend cells by inverting tube several times. Fill 1-ml syringe with attached 27-G, ½-in. needle.

13. Hold a mouse with one hand, use the other hand to slide needle 5 to 10 mm subcutaneously (Donovan and Brown, 2006c), and inject 100 μl cell suspension. Watch for appearance of a "bleb."

    Holding a mouse with a single hand requires some practice: use thumb and index finger to hold the neck and use ring finger and little finger to press down the hind legs. This will result in a much tightened abdominal skin, which is critical for successful subcutaneous injection.

    Failure to insert needle far enough will result in leakage of tumor suspension when mice massage the area after injection. If no clear "bleb" results, sacrifice mouse and use a new one.

14. Gently withdraw needle and place mouse back in cage.

    Do not let the syringe with cell suspension sit undisturbed on bench for more than 5 min, because the cells can settle quickly.

Randomize mice and follow up

15. Ear tag mice (*UNIT 1.5*; Donovan and Brown, 2006b) to blind the experiment and randomize among cages.

16. Observe mice for tumor growth. Use calipers to measure perpendicular tumor diameters.

    Tumors should become palpable in 5 to 10 days.

MOUSE MODEL OF MULTIPLE PULMONARY TUMOR METASTASES

BASIC PROTOCOL 2

The pulmonary metastasis model is the other widely used method for evaluating therapy in many tumor models, including B16 melanoma. Since essentially all tumor cells that "take" upon intravenous tumor injection are found in the lungs, the term pulmonary metastasis is widely used even though every resulting pulmonary nodule is technically an independent "primary" tumor rather than a true metastasis. The typical dose used is 2×10^5 cells/mouse, which will yield between 50 and 250 pulmonary nodules visible on the lung surface. Since the melanin in B16 does not bleach like the rest of the pulmonary tissues, the tumor nodules can be easily visualized after bleaching of the extracted lungs in Fekete's solution (see Reagents and Solutions). There is always a fraction of nodules that are amelanotic ("white"); therefore, careful counting is required in order not to underestimate tumor burden. For non-pigmented tumor, India ink is injected into the lungs to create a color contrast.

Intravenous injections (tail vain) are difficult and require extensive practice. It is recommended that no "real" experiments be performed until investigators are comfortable with the technique, since the inexperienced injector is likely to introduce inconsistent tumor take among mice.

Materials

B16 culture, 50% to 80% confluent (see Support Protocol 1)
PBS or HBSS (Life Technologies)
6- to 12-week old female C57BL/6 mice

100-μm cell strainer (BD Falcon)
Heat lamp
1-ml disposable syringes and 27 G, ½ in. needles

Additional reagent s and equipment for preparing cell suspension (Basic Protocol 1), counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997a),

counting viable cells by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b), restraint of mice (*UNIT 1.3*; Donovan and Brown, 2006a), ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b), intravenous injections (*UNIT 1.6*; Donovan and Brown, 2006c), euthanasia of mice (*UNIT 1.8*; Donovan and Brown, 2006d), and enumeration of lung metastases (Support Protocol 2)

Prepare B16 cells

1. Perform steps 1 to 8 of Basic Protocol 1 to prepare cell suspension.

   Take special care to remove clumps by straining the trypsinized cells just before injection. Clumps can kill mice upon intravenous injection by causing embolisms.

2. Adjust cell concentration to 4×10^5 cells/ml in ice-cold PBS or HBSS.

Inoculate mice

3. Relocate work area to the facility where 6- to 12-week-old female C57BL/6 mice are kept. Maintain cells on ice.

   Inject cells as quickly as possible after preparation; viability slowly decreases over time, even on ice.

4. Warm mice by placing open cage under heat lamp for 2 to 3 min, until tail veins are dilated.

5. Thoroughly resuspend cells by inverting tube several times. Quickly fill 1-ml syringe with B16 cells and attach a 27-G, ½-in. needle.

   Failure to resuspend will result in steadily decreasing numbers of tumor cells injected. Avoid air bubbles in the syringe.

6. Immobilize mouse in a restraint that allows the tail to protrude (*UNIT 1.3*; Donovan and Brown, 2006a) and inject 0.5 ml cell suspension intravenously into dilated tail vein (*UNIT 1.6*; Donovan and Brown, 2006c).

   The tail veins run laterally from the clearly visible central tail artery, into which injection is difficult. Start towards the tip of the tail; if the injection fails to enter easily, the needle is not inside the vein; do not try to force it in, but try again by moving closer to the base of the tail.

7. Resuspend cells before each syringe refill.

Randomize mice and follow up

8. If mice have not been pretreated, ear tag mice (*UNIT 1.5*; Donovan and Brown, 2006b) and randomize mice to blind the experiment.

9. Euthanize mice by CO_2 asphyxiation (Donovan and Brown, 2006d) on day 16 to 18 after tumor inoculation, and enumerate lung metastases (see Support Protocol 2).

   Alternatively, follow mice for survival.

   Untreated mice will begin to die of pulmonary congestion caused by tumor, starting around day 21.

BASIC PROTOCOL 3

INDUCTION OF SPONTANEOUS TUMOR IN TRANSGENIC MICE

In recent years, genetic modifications in mice have provided new opportunities for investigators to study spontaneous tumors that can better mimic human cancers. Some of the early versions of mouse models overexpress oncogenes under the control of tissue-specific promoters (Stewart et al., 1984; Hanahan, 1985; Greenberg et al., 1995). The more advanced models combine the interruption of tumor suppressor genes (Donehower et al., 1992; Aguirre et al., 2003; Hingorani et al., 2005) and inducible tissue-specific

"knock-in/out" technology to develop tumors in a controlled manner. The latter models are more suitable for therapeutic studies. One important note for immunological studies is that mouse strain is critical for any MHC-restricted intervention. In order to avoid the complications caused by any differences in minor histocompatability during adoptive cell transfer, complete back-cross ($N>10$) to the designated strain is preferred. In this unit, the authors demonstrate the induction of spontaneous cutaneous tumor in Tyr:CreERT2/BRAFhV600E *CA/CA*/PTEN *flox/flox* transgenic mice.

Materials

4-hydroxytamoxifen (4-HT, > 70% z-isomer; Sigma),
Dimethylsulfoxide (DMSO, Sigma)
4- to 5-week-old Tyr:CreERT2/BRAFhV600E *CA/CA*/PTEN *flox/flox* transgenic mice
Nair hair remover

Brown-colored non-transparent microcentrifuge tubes
Hair clipper (Wahl, Model 8761)
Sterile swabs
Fine-tip paint brush
Calipers

Prepare 4-HTsolution

1. Dissolve 1.9 mg/ml of 4-HT in DMSO to make a 5 mM solution. Prepare 50 to 100 µl aliquots in dark microcentrifuge tubes to avoid light degradation and store in −20°C freezer.

Induction of tumor

2. Relocate work area to facility where Tyr:CreERT2/BRAFhV600E *CA/CA*/PTEN *flox/flox* transgenic mice are housed.

3. Shave the lower back of mice and apply a thin layer of Nair hair remover on the shaved skin with a sterile swab.

   Because spontaneous tumors can occasionally occur without 4-HT treatment after 12 weeks of age in this strain of transgenic mice (Tyr:CreERT2/BRAFhV600E CA/CA/PTEN flox/flox), 4- to 5-week old mice are optimal for tumor induction by 4-HT.

4. After 5 min of Nair application, wet a paper towel with warm water and wipe off Nair. Repeat until the lotion is completely cleared from the skin.

5. Drop 2 µl of 4-HT solution onto the clean skin with a pipettor 10-µl pipet tip and use a fine tip paint brush to evenly spread the 4-HT in a 5-mm × 5-mm area.

Monitor tumor growth

6. After tumor development (1 to 2 months), monitor the growth by measuring perpendicular tumor diameters with calipers.

TUMOR PROTECTION USING GM-CSF-TRANSDUCED WHOLE-CELL VACCINE (B16.GM-CSF)

BASIC PROTOCOL 4

It is difficult to induce reliable protection against aggressively growing tumors, such as mouse B16 melanoma challenge, by vaccination with irradiated tumor, even when admixed with *Corynebacterium parvum.* However, robust protection can be obtained by vaccinating with tumor that is retrovirally transduced to secrete high levels of GM-CSF (Dranoff et al., 1993). Although B16.GM-CSF will still grow upon injection, vaccination

with irradiated cells will induce a T cell–dependent protection against wild-type B16.F10. It is unknown what antigens are targets of the immune protection, and the involvement of eosinophils and macrophages has been implicated (Hung et al., 1998). The following protocol describes the use of B16.GM-CSF for protection against B16 challenge in the authors' laboratory. Additional results suggest that it may also be possible to impact the growth of established tumors by vaccinations with irradiated B16.GM-CSF, especially in conjunction with anti-CTLA-4 antibody (van Elsas et al., 1999b). The addition of this antibody, which presumably abrogates T cell–inhibitory signaling through the CTLA-4 receptor, enhances protection and also allows for the induction of vitiligo, which does not routinely result when vaccinating with B16.GM-CSF alone.

When using a whole-cell vaccine, it becomes of greatest importance to ensure that tumor cells are free of mycoplasma, since vaccination with mycoplasma-contaminated cells and subsequent challenge with mycoplasma-contaminated cells could result in impressive mycoplasma-specific tumor rejection.

Materials

B16.GM-CSF culture, 50% to 80% confluent (see Support Protocol 1; procedure for preparing these cells is the same as for the B16 cells used in Basic Protocol 1)
PBS or HBSS (Life Technologies), ice cold
6- to 12-week old female C57BL/6 mice
Wild-type B16.F10 culture (ATCC, cat. no. CRL-6475), 50% to 80% confluent (Support Protocol 1)

γ irradiator
Calipers

Additional reagents and equipment for preparing cell suspension (Basic Protocol 1, steps 1 to 8), injection of cell suspension into mice (Basic Protocol 1, steps 11 to 16), and ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b)

Prepare B16.GM-CSF vaccine

1. Perform steps 1 to 8 of Basic Protocol 1 to prepare B16.GM-CSF cell suspension.
2. Adjust cell concentration to 1×10^7/ml in ice-cold PBS or HBSS.
3. Irradiate cells in a γ irradiator with a dose of 50 Gy.

Vaccinate mice

4. Relocate work area to the facility where 6- to12-week old female mice are housed. Maintain cells on ice.
5. Inject 100 μl (1×10^6 cells) of the irradiated B16.GM-CSF subcutaneously, as described in Basic Protocol 1, steps 11 to 14.

Challenge mice

6. At a time point 2 to 3 weeks after vaccination, challenge mice with 1×10^5 wild-type B16.F10 as described in Basic Protocol 1.

Randomize mice and follow-up

7. Ear tag mice (*UNIT 1.5*; Donovan and Brown, 2006b) to blind the experiment and randomize over cages.
8. Observe mice for tumor growth. Use calipers to measure perpendicular tumor diameters.

INDUCTION OF B16 MELANOMA PROTECTION BY RECOMBINANT VACCINIA VIRUS (rVV) VACCINE

Currently, the two tumor vaccines that have induced the most reliable protection of mice from lethal B16 challenge are rVV encoding the melanoma differentiation antigen (MDA), mTRP-1, and irradiated B16 expressing GM-CSF (Hung et al., 1998; Overwijk et al., 1999a). Although immunization with rVVhgp100 induces high levels of gp100-specific cytotoxic T lymphocytes (CTL), this regimen is completely ineffective in preventing B16 growth upon intravenous or subcutaneous challenge. However, adoptive transfer of in vitro cultured, gp100-reactive CTL can greatly reduce the number of lung metastases upon subsequent intravenous B16 challenge (Overwijk et al., 1998).

The following protocol describes the induction of tumor protection by vaccination with rVVmTRP-1. All viruses mentioned in this unit can be obtained through Dr. Nicholas Restifo at the Surgery Branch, NCI, NIH. Using this approach, vitiligo is also induced in essentially every vaccinated mouse, and can initiate anywhere on the body, with some apparent preference for the abdomen. Depigmentation should be scored blindly and compared with control mice receiving two injections of a control rVV encoding an irrelevant antigen. A slightly lighter shade of black or brown is not vitiligo, as this can occur at random even in untreated mice. Vitiligo manifests itself as clear, usually sharply demarcated white spots or bands, often bilaterally distributed over the body. Mice typically do not turn completely white, though an additional booster injection of rVVmTRP-1 may enhance the degree of depigmentation. Mice receiving only one vaccination of rVVmTRP-1 never develop vitiligo and are not protected from tumor challenge.

Safety Concerns Regarding the Use of Recombinant Vaccinia Viruses

Vaccinia virus (VV) and adenovirus have a very broad host range, infecting virtually all mammalian cells, including human. Everyone born before the early to mid-1970s has been vaccinated with the smallpox vaccine, which provides a long-lasting protection against systemic viremia upon VV infection. Most people are continually exposed to adenovirus, which is present in the upper respiratory tract of a large portion of healthy individuals where it ordinarily does not cause pathology. However, it is important to realize that, even in individuals who have been repeatedly boosted, a limited infection does occur upon dermal scarification with VV, as evidenced by a localized pustule and tender draining lymph nodes. Evidence is accumulating that mucosal immunity to vaccinia may be weak, so protection from infection through nose, mouth, and eyes may be particularly poor. In addition, wild-type adenovirus causes significant numbers of respiratory tract infections and even deaths, especially in infants and the elderly. Although rVV, rFPV, and rAd used in vaccine approaches are all selected and/or modified low-virulence viral strains that have been administrated safely to humans in multiple clinical trials, the growing list of modifications artificially introduced in viral genomes makes them unpredictable agents. This becomes especially important when using viruses encoding human genes or their closely related mammalian counterparts. The effects of viruses encoding MDA on healthy individuals are largely unknown, but the real possibility exists that accidental exposure induces immunity to MDA, with vitiligo as a potential outcome. This is more than a theoretical risk, especially with the use of rVVmTRP-1, which induces irreversible vitiligo in mice. The greatest care should be taken when using recombinant viruses, especially those encoding mammalian “self” antigens. Wear gloves, protective eye wear, and use utmost precautions when injecting animals. Do not recap needles, and instantly discard used syringes and needles in designated biohazard sharp boxes. VV and FPV, especially, are quite stable viruses that retain infectiousness after lyophilization or prolonged exposure to room temperature. Do not leave virally contaminated tubes, pipets, or pipet tips in the laminar flow cabinet or exposed in the biohazard waste box;

use bleach to destroy the viruses. Do not flush leftover viral preparations down the drain before bleach treatment. As an additional precaution, those who intend to work with rVV should request a vaccination with smallpox vaccine regardless of whether they were ever vaccinated before. Virtually every animal facility has rules and regulations regarding the use of recombinant organisms in animals: be sure to contact your animal facility safety personnel. Handle recombinant viruses with respect.

Materials

6- to 12-week old female C57BL/6 mice
rVVmTRP-1 and control rVV encoding an irrelevant antigen (available from the authors; contact Dr. Nicholas Restifo at the Surgery Branch, NCI, NIH), prepared for intravenous injection (Support Protocol 4)
Wild-type B16.F10 culture, 50% to 80% confluent (Support Protocol 1)

Additional reagents and equipment for immunization of mice with recombinant viral vectors (see Support Protocol 4), subcutaneous challenge with B16 cells (Basic Protocol 1), and ELISA detection of antibodies against MDA (Support Protocol 5)

Immunize mice

1. Inject 6- to 12-week old female C57BL/6 mice intravenously with 1×10^7 pfu rVVmTRP-1 as described in Support Protocol 4. Also immunize mice with a control rVV encoding an irrelevant antigen.

   Prepare rVV for intravenous injection according to instructions in Support Protocol 4.

2. Two to 3 weeks later, boost mice intravenously with 1×10^7 pfu rVVmTRP-1 or control rVV.

Monitor for vitiligo

3. Between 2 and 3 weeks after the second immunization with rVVmTRP-1, look for clearly developed depigmentation.

Challenge with B16 tumor

4. As soon as depigmentation is apparent in all mice (i.e., typically 2 to 4 weeks after the booster injection), challenge mice subcutaneously with wild-type B16.F10 melanoma and follow tumor growth (see Basic Protocol 1).

Monitor serological response

5. Collect serum from immunized mice at any time for testing by ELISA (see Support Protocol 5).

BASIC PROTOCOL 6

ADOPTIVE TRANSFER OF TUMOR ANTIGEN–SPECIFIC T CELLS TO TREAT ESTABLISHED TUMOR

Adoptive transfer of tumor antigen-specific T cells to treat established tumor in mice have been demonstrated by the authors' research team and others in the field of cancer immunotherapy (Overwijk et al., 2003; Chinnasamy et al., 2010; Kerkar et al., 2010; Kochenderfer et al., 2010). This experimental model has been directly translated into several clinical trials (Dudley et al., 2002; Kochenderfer et al., 2013; Phan and Rosenberg, 2013) as well as adapted to study the mechanisms of adoptive immunotherapy and provide insights to improvement of therapeutic efficacy.

To be considered as an established tumor, vascularization is one of the criteria. In subcutaneous models, vascularization occurs after tumor size reaches 5 to 10 mm in diameter. In pulmonary metastasis models, a 7-day tumor growth period prior to treatment is much

preferred over a 3-day tumor growth period. In recent years, a bioluminescence imaging system using a cooled CCD camera is utilized to monitor systemically growing tumors expressing luciferase (see Support Protocol 3). The treatment schedule can therefore be determined based on the imaging results.

T cells used for adoptive transfer can be from various sources: freshly isolated lymphocytes either from spleens or lymph nodes; FACS-sorted or antibody column–enriched lymphocyte subsets; or ex vivo cultured T cells with antigen stimulation or retroviral transduction. To ensure the quality of these T cells, it is recommended to test their functions in vitro prior to adoptive transfer. The viability of cells should be greater than 50%, since the clumps of dead cells can cause acute pulmonary embolisms in mice. In 6- to 8-week old female C57BL/6 mice, a single dose of intravenously administered cells should not exceed 1×10^8 cells. A typical dose is 1×10^6 cells/mouse, and when titrating the cell numbers, a log increment is recommended to allow measurable differences in tumor treatment results.

The current standard protocol for adoptive immunotherapy in B16 melanoma model also requires sublethal total body irradiation and cytokine administration prior to and after adoptive cell transfer, respectively (Gattinoni et al., 2005), as well as vaccination to activate antigen specific T cells (with the exception of using CAR transduced T cells; van Elsas et al., 2001). Although alternatives have been identified to replace these requirements (Wrzesinski et al., 2007; Muranski et al., 2008; Dudda et al., 2013), the authors suggest using the standard protocol as a control.

Materials

T cells in 24-well plates (see Support Protocols 6 to 9)
PBS or HBSS (Life Technologies), ice cold
Tumor-bearing mice (Basic Protocols 1 to 3)
Recombinant viral vaccines (optional; see Basic Protocol 5)
Recombinant human IL-2 (optional)

Multichannel pipettor with 1000 µl (P-1000) pipet tips
100-ml reagent basins, sterile
50-ml conical centrifuge tubes
Refrigerated centrifuge (such as Sorvall RC4)
γ irradiator for small animals (optional)
Heat lamp
3-ml and 1-ml disposable syringes with 27-G, ½-in. needles
Calipers

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997a) and determining viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b), restraint of mice (*UNIT 1.3*; Donovan and Brown, 2006a), ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b), intraperitoneal injection of mice (*UNIT 1.6*; Donovan and Brown, 2006c), euthanasia by CO_2 asphyxiation (*UNIT 1.8*; Donovan and Brown, 2006d), bioimaging (Support Protocol 3), and enumerating tumor nodules (Support Protocol 2)

Prepare T cells

1. Harvest T cells in culture medium from 24-well plates using a multi-channel pipettor and collect the cell suspension in a reagent basin. Transfer the cells into 50-ml conical centrifuge tubes.

   One confluent well of a 24-well plate typically yields 5×10^5 to 2×10^6 cells.

2. Centrifuge the cell suspension for 10 min at 663 × *g* (1500 rpm using Sorvall RC4), 4°C. Discard the supernatant and resuspend T cells in ice-cold HBSS or PBS, aiming for a final concentration of twice the designated cell numbers per mouse per ml.

   If a large amount of debris is present in culture, purify T cells on a Ficoll gradient according to the manufacturer's protocol.

3. Count cells using trypan blue to exclude dead cells (*APPENDIX 3A & 3B*; Strober, 1997a,b).

4. Dilute cells to desired concentration and keep on ice.

Transfer T cells

5. Relocate work area to the facility where 6- to 12-week old female mice are housed.

6. Randomize tumor-bearing mice (5 to 10 mm in diameter for subcutaneous tumor, day 7, or desired imaging size for systemic tumor) by pooling in a large cage.

7. *Optional:* Irradiate mice using γ irradiator with a sublethal dose to deplete endogenous lymphocytes.

   In C57BL/6 mice, 5 Gy is the standard dose. Irradiation of mice can be done one day before cell transfer.

8. Warm mice by placing under heat lamp for 2 to 3 min until tail veins are dilated.

9. Resuspend T cells before injection by repeatedly inverting tube. Fill 1-ml syringe attached to 27-G, ½ in. needle with cell suspension.

   When vaccinations are required, mix less than 10 μl/mouse (i.e.,1–2 × 10^7 pfu of rVV virus/mouse; see Basic Protocol 5) into the cell suspension immediately prior to intravenously injection (optional).

10. Immobilize tumor-bearing mice using a restraint that allows tail to protrude (*UNIT 1.5*; Donovan and Brown, 2006b) and inject 0.5 ml of T cell suspension intravenously into tail vein.

    When injecting large numbers of cells (i.e., > 5 × 10^6), inject slowly to prevent embolisms.

    Mice without any treatment or injected with PBS or HBSS serve as controls.

11. Inject 60,000 to 600,000 IU rhIL-2 in 0.5 ml of PBS intraperitoneally using a 1-ml or 3-ml syringe and 27-G, ½-in. needles 1 to 2 times a day for 3 days.

    Mice should be closely monitored during the first week after cell transfer. If mice show any signs of distress or weight loss, adding soft food and hydrating gel in the cage can help their recovery.

Evaluate tumor burden

12. Measure perpendicular tumor diameters of subcutaneous tumors three times per week using calipers. For systemic growing tumors, use bioimaging weekly (see Support Protocol 3). For pulmonary metastases, euthanize mice by CO_2 asphyxiation (*UNIT 1.8*; Donovan and Brown, 2006d) 16 to 18 days after tumor inoculation and enumerate the tumor nodules (see Support Protocol 2). Alternatively, follow mice for survival.

BASIC PROTOCOL 7 CLONING HLA-RESTRICTED ANTIGEN-SPECIFIC T CELL RECEPTORS FOR CLINICAL USE

HLA-transgenic mice are useful tools to generate T cells against antigens that are "self" in human, but "non-self" in mice. Because these T cells are not eliminated during the

thymic negative selection, the affinity of their TCRs can be higher than those on human T cells. During the past decade, the authors have successfully identified several T cell receptors with high affinity to human tumor antigen in the context of HLA-A*0201 (Johnson et al., 2009; Parkhurst et al., 2009; Chinnasamy et al., 2011), and translated them directly into clinical trials.

Target antigens and their HLA-restricted epitopes need to be carefully selected prior to using this protocol. An ideal target antigen should be abundantly expressed in tumor cells and can be presented by HLA molecules on the cell surface. Although an MHC-binding algorithm is commonly used to predict binding affinity of peptides derived from an antigen, the most reliable method is the direct detection of peptide/MHC complex on tumor cells by mass spectrometry. Tumor specificity is also a very important concern for target selection. Two categories of proteins are currently on the list: (1) proteins that are only expressed in non-life-threatening tissues, such as melanocyte differentiation antigens and tumor-testis antigens; (2) proteins that are mutated only in tumors and mutated peptide/MHC complexes presented on tumors.

Specificity of the cloned TCR needs to be thoroughly tested before any consideration of clinical use. The panel of target cells should include not only tumors expressing the antigen and corresponding MHC, but also tumors expressing the antigen without corresponding MHC as well as tumors expressing neither the antigen nor the MHC.

Materials

Peptide of interest or recombinant viral vaccines (see Basic Protocol 5 for the vaccines)
HBV-core helper peptide 128-140: TPPAYRPPNAPIL (a PAN MCH class II helper peptide)
DSMO (Sigma)
Sterile HBSS or PBS (Life Technologies)
Incomplete Freund's adjuvant (Sigma)
6- to 8-week old female HLA transgenic mice (some HLA transgenic mice are commercially available from The Jackson Laboratory or Taconic)
Complete medium (CM; see recipe)
Ultrapure lipopolysaccharide (LPS) from *E. coli* 0111:B4 strain (InVivogen), prepared at 5 mg/ml in sterile water according to manufacturer's manual.
Recombinant human IL-2
Target cells for testing antigen specificity (see annotation to step 16, below)
Peptide/HLA tetramers (custom-made at NIH Tetramer Core Facility, Emory University, Atlanta, Ga)
FACS buffer (see recipe)
RNAlater solution (Life Technologies)
RNeasy Mini Kit (Qiagen)
SMART cDNA synthesis kit (Clontech)
SMARTer RACE kit (Clontech)
Gene-specific primers for mouse T cell receptor: α-chain ggctactttcagcaggagga, β-chain aggcctctgcactgatgttc
1.2% agarose gel with ethidium bromide (*UNIT 10.4*; Voytas, 1991)
Zymoclean Gel DNA recovery kit (Zymo Research)
TA cloning kit (Life Technologies)
TOP 10 *E. coli* (Invitrogen)
LB liquid medium and LB agar plates (e.g., *UNIT 2.17*; Baral et al., 2013)
Appropriate selection antibiotic
Plasmid DNA Miniprep kit (Qiagen)
DNA sequencing facility

GeneArt Gene synthesis service (Life Technologies)
MSGV1 retroviral vector with a linker sequence encoding the foot and mouth disease picornavirus 2A "self-cleaving" site separating the two (available from the authors; contact Dr. Nicholas Restifo at the Surgery Branch, NCI, NIH)

24- and 48-well culture plates
Dremel MultiPro 3955 variable speed kit with a 1/8-in. drill
1-ml syringes
19-G and 27-G, ½-in. needles
70% isopropyl alcohol prep (Webcol; Kendall Healthcare)
162-cm^2 tissue culture coated flasks
50-ml conical tubes
Sterile dissecting tools
γ irradiator
FACS tubes
96-well round-bottom plates

Additional reagents and equipment for footpad injection of (Donovan and Brown, 2006c), subcutaneous injection of mice (Basic Protocol 1), preparation of single-cell suspension from mouse spleens (Support Protocol 6), euthanasia of mice (*UNIT 1.8*; Donovan and Brown, 2006d), counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997), determining viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b), FACS (Chapter 5 in this manual), antigen-specific IFN-γ production (Support Protocol 7; optional), and agarose gel electrophoresis (*UNIT 10.4*; Voytas, 1991)

Prepare peptide-adjuvant emulsion

1. Dissolve immunization peptide in DMSO at 20 mg/ml and dissolve HBV-core helper peptide in DMSO at 24 mg/ml.

   This solution can be stored at 4°C for several months. If the peptides do not completely dissolve in DMSO, use as a suspension instead of a solution, ensuring that it is mixed well immediately before step 2.

2. Mix 50 μl of immunization peptide solution, 50 μl of HBV-core helper peptide/DMSO solution, and 400 μl of PBS in a microcentrifuge tube.

   It is not recommended to use less than 1 ml of the final volume (0.5 ml of peptide and 0.5 ml of IFA) to prepare peptide/IFA using the drill-mix method. The advantage of using a drill instead of syringes to prepare the emulsion is saving time; however, one could lose up to 20% to 30% of the emulsion during transfer of emulsion to syringe. In general, one preparation should be enough to immunize five mice.

3. Prepare the emulsion.

   a. Place a 48-well plate on ice and add 500 μl of incomplete Freund's adjuvant (IFA) to one well.
   b. Install a clean 1/8-in. drill into the head of the Dremel. Hold the Dremel with one hand, insert the drill tip in the well and start the speed at 1 to 2.
   c. Meanwhile, pipet 0.5 ml of the peptide solution (from step 2) into the well, then slowly drip in IFA over a period of about 10 to 20 sec. After all the peptides are added into the well, increase the speed to 4 to 5 and carefully stir the mixture for 30 sec.
   d. Test the formation of emulsion by dropping 20 μl of the mixture onto water in a separate beaker. If the mixture does not spread out and forms an immiscible drop on the water surface, the emulsion is ready for use. Otherwise, repeat mixing at high speed for another 30 sec.

Two to three repeats should be sufficient.

4. Slowly fill a 1-ml syringe attached to a 19-G needle with the emulsion, making an effort to avoid any air bubbles. Change the needle to 27-G, ½ in., and place the syringe on ice.

Immunize mice

5. Relocate work area to the facility where 6- to 12-week old female HLA transgenic mice are housed.

   The immunization procedure requires two people. One holds the mice and one injects.

6. Wipe foodpads and tail with 70% isopropanol prep prior to injection. Inject 25 μl of emulsion into each foodpad (*UNIT 1.6*; Donovan and Brown, 2006c) and 50 μl subcutaneously into the base of the tail (see Basic Protocol 1 for subcutaneous injection).

   Inflammation of footpads is minimal using IFA; however, mild swelling may occur in some cases 5 to 7 days post injection. Be sure to monitor the mice and provide soft food and hydrating gel in the cage. The booster immunization may be only given at the base of the tail if the swelling persists.

7. Repeat the same immunization procedure 2 weeks later.

   If using recombinant virus, see Support Protocol 4.

Prepare antigen-presenting cells

8. At a time point 5 days after the second immunization, harvest one spleen from a non-immunized HLA transgenic mouse and make a single-cell suspension (see Support Protocol 6). Resuspend the splenocytes in 30 ml of CM containing 1 μg/ml of LPS, plate the cells in a 162-cm^2 tissue culture flask, and incubate for 2 days in a 37°C, 5% CO_2 humidified incubator.

   These LPS-stimulated lymphoblasts are used as antigen-presenting cells (APCs) in stimulating T cells. Extra cells can be frozen in liquid nitrogen for future use.

9. At a time point 7 days after the second immunization, collect LPS-activated lymphoblasts into a 50-ml conical tube. Wash once with 50 ml CM by centrifuging 5 min at 663 × *g*, 4°C, remove supernatant, and resuspend in CM at 3×10^6 cells/ml.

10. Add immunization peptide to the activated lymphoblasts at a concentration of 1 μg/ml or less (see step 1 for preparation of stock) and put the cells in a 37°C/ 5% CO_2 incubator for 2 to 4 hr with occasional mixing.

    If the HLA transgenic mice have unstable MHC on the surface due to the lack of binding site of β2-microglobulin, 10 μg/ml of human β2-microglobulin can be added to the culture during this peptide pulse. Peptide concentration can be as low as 0.01 μg/ml depending on its binding affinity to HLA molecules. If the affinity is unknown, start at 1 μg/ml.

Stimulate bulk T cells

11. During peptide pulsing, euthanize the immunized mice by cervical dislocation (*UNIT 1.8*; Donovan and Brown, 2006d) and immediately harvest spleens, popliteal lymph nodes, and inguinal lymph nodes into 50-ml conical tubes containing 10 ml of sterile PBS. Properly discard animal carcasses.

    Spray the mouse carcass with 70% isopropyl alcohol and use sterilized instruments for tissue harvesting. If multiple mice are used for one peptide immunization, tissues from individual mice should be collected separately. Spleens and lymph nodes should also be collected in separate tubes. However, once the single-cell suspensions are prepared, splenocytes and lymph node cells from a single mouse can be combined for stimulation.

12. Make single-cell suspension according to Support Protocol 6. Count viable cells using a hemacytometer (*APPENDIX 3A*; Strober, 1997a) and trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b). In a 50-ml conical tube, adjust cell concentration to 3×10^6 cells/ml in CM and place it on ice.

13. Irradiate peptide-pulsed APCs with 30 Gy of γ radiation. Wash the cells with CM three times as described in step 9 and resuspend the cells in the same volume of CM as used in peptide pulsing.

14. In a 24-well plate, add 1 ml of peptide-pulsed APCs (3×10^6 cells) and 1 ml of immunized splenocytes or lymph node cells (3×10^6 cells) to each well. Incubate in a 37°C, 5% CO_2 incubator for 7 to 10 days.

15. Every 7 to 10 days, restimulate the cells under the same conditions except add 60 IU/ml of IL-2 to the culture medium.

Test for antigen specificity

This test can be done 7 days after each round of ex vivo stimulation.

16. During the ex vivo peptide stimulation, culture various target cells.

    *The target cells may include: LPS-activated transgenic splenocytes, HEK 293 cells expressing HLA restriction elements and antigen, or tumor cells. In the case of HLA-A*0201, T2 cells can be used as target cells.*

17. After 7 days of ex vivo peptide stimulation, perform either the tetramer binding assay or antigen-specific IFN-γ production assay.

    a. If peptide/HLA tetramers are available, transfer 100 μl of cell suspension from each well into an individual FACS tube and add 2 μl (or manufacturer's recommended amount) of target peptide/HLA tetramer or control tetramer. Incubate on ice for 20 min.
    b. Wash the cells once with FACS buffer. Resuspend the cells in 200 μl of FACS buffer and proceed to FACS.
    c. If tetramers are not available, test T cell functions by antigen-specific IFN-γ production assay using Support Protocol 7. Each well in the 24-well plate is tested separately. When setting up the co-culture, duplicated wells are preferred.

    Due to a limited number of effector cells, T cells are not counted during the co-culture. By adding 100 μl of cell suspension to each well of the 24-well plate, 10×10^4 cells can be placed in one well of a 96-well round-bottom plate. Save tetramer-positive and co-culture cell pellets by adding 200 μl of RNAlater solution (Life Technologies) to each well and storing at –20°C.

Clone antigen-specific T cells

18. Harvest T cells that test positive for antigen reactivity from bulk stimulations and make a single-cell suspension at 10, 3, 1 and 0.3 cells/100 μl of CM containing 60 IU/ml IL-2.

19. Prepare LPS-activated lymphoblasts and pulse with peptide (irradiated) as described in steps 8 to 10. Count viable cells using a hemacytometer (*APPENDIX 3A*; Strober, 1997a) and trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b). Adjust the cell concentration to 5×10^5 cells/ml with CM containing 60 IU/ml IL-2.

20. Add 100 μl of T cells (10, 3, 1, and 0.3 cells/well) to each well of a 96-well round bottom plate and 100 μl of peptide-pulsed irradiated APCs (4×10^4 cells/well) to the same well. Incubate in a 37°C, 5% CO_2 humidified incubator for 12 to 14 days.

21. Monitor the growth of T cell clones by observing the cells under a microscope. Transfer any proliferating T cells first into a 96-well plate and then a 48-well plate.

    T cell proliferations usually occur 5 to 7 days after antigen stimulation.

22. Test proliferating clones according to steps 16 to 17 before molecular cloning.

    Save tetramer-positive and co-culture cell pellets by adding 200 μl of RNAlater solution to each well and storing at –20°C. These materials can be used for molecular cloning.

Clone antigen-specific T cell receptor

23. Isolate total RNA from T cell clone using Qiagen RNeasy Mini Kit according to the manufacturer's instructions.

    The total T cell numbers required for molecular cloning can be as low as a few thousand. If positive cells are not found during the limiting-dilution cloning, the cell pellets saved after each positive testing can be used for RNA isolation.

24. Prepare 5′ RACE-ready cDNA from total RNA using the Clontech SMART (Switching Mechanism at 5′ End of RNA Template) cDNA synthesis kit according to the manufacturer's instructions.

25. Perform 5′ RACE (Rapid Amplification of cDNA Ends) using Clontech SMARTer RACE kit according to the manufacturer's instruction with the gene specific primers for mouse T cell receptor.

26. Run the RACE PCR products on 1.2% agarose gel electrophoresis (*UNIT 10.4*; Voytas, 1991) and purify any distinctive bands appearing at 800 to 900 base pairs using the Zymoclean Gel DNA recovery kit according to the manufacturer's instructions.

27. Ligate freshly isolated 5′ RACE PCR products into a TA cloning vector using the Invitrogen TA cloning kit and transform into TOP 10 *E. coli* (Invitrogen) according to the manufacturer's instructions.

28. Grow bacteria culture from plated colonies in 1.2 ml of selection LB medium for plasmid DNA isolation using Qiagen Plasmid DNA Miniprep kit.

29. Sequence RACE product in plasmid DNA using the sequencing primers provided with the TA cloning kit to determine TCR 5′ sequences.

30. Use the 5′ sequencing information to design 5′ gene specific primers and 3′ gene specific primers for TCR α-chain (tcaactggaccacagcctcagc) and β-chain (tcatgaattctttcttttgaccatagcc) to PCR-amplify full-length TCR using 5′ RACE-ready cDNAs and Clontech Advantage 2 HF Taq polymerase (following the manufacturer's recommendation). Use the following PCR conditions:

1 cycle:	1 min	95°C
35 cycles:	30 sec	95°C
	3 min	68°C
1 cycle:	3 min	68°C
1 cycle:	indefinitely	4°C.

31. Repeat steps 26 to 29 and ascertain that the full-length TCR PCR products are 1500 to 1600 bp in length.

32. Compare the full length TCR sequences to the database published at *http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=mouseTcR* and determine the TCR nomenclatures. Exclude any sequences containing premature stop codons.

33. Synthesize codon optimized TCR sequences using GeneArt Gene synthesis service and clone the α-chain and β-chain genes into a MSGV1 retroviral vector with a linker sequence encoding the foot-and-mouth disease picornavirus 2A "self-cleaving" site separating the two.

This viral vector can be obtained from Dr. Nicholas Restifo at Surgery Branch, NCI. Retrovirus encoding the TCR can be made by transfecting Plat-E cells(see Support Protocol 10). Retrovirally transduced human T cells (see Support Protocol 10) are tested again for tumor recognition before applying for clinical trials.

SUPPORT PROTOCOL 1

GROWING B16 MELANOMA

Many laboratories carry B16 lines; however, it is important to note that B16 tends to be an unstable cell line that has many variants among laboratories. Even within one laboratory, B16 can change quickly over time. Changes that have been observed include dramatic differences in the degree of pigmentation, minimum tumorigenic dose, metastatic potential, antigen expression, and recognition by CTL. Pigmentation, which may change from week to week, does not appear to clearly correlate with MDA expression or recognition by CTL; however, it is still important to freeze a large number of vials of the B16 line that "works," and regularly thaw a new vial. B16 freezes and thaws well; trypsinized B16 cells can be washed in complete medium and frozen in 90% FBS/10% DMSO. Regardless of the source, every new tumor cell line that enters the laboratory should be tested for mycoplasma; these microorganisms are prevalent and may significantly influence in vitro and in vivo results, particularly tumor "take" upon injection. In addition, any murine tumor lines to be used in mice should be subjected to a murine pathogen test such as the molecular testing of biological material (MTBM) or murine antibody production (MAP) test. B16.F10 and other sublines can be obtained from American Type Culture Collection (ATCC; see *APPENDIX 5*) or the DCTDC Tumor Repository at the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, Maryland). The DCTDC catalog contains additional information on B16.F10 melanoma and other sublines.

Materials

Cryopreserved vial of B16.F10 (ATCC, cat. no. CRL-6475; or other B16 line, e.g., B16.GM-CSF)
DMEM medium (see recipe), ice cold

15-ml conical centrifuge tubes (e.g., BD Falcon)
Refrigerated centrifuge (such as Sorvall RC4)
162-cm^2 tissue culture–coated flasks

Additional reagents and equipment for counting cells using a hemacytometer (*APPENDIX 3A*; Strober, 1997a) and determining viable cells by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b)

Thaw and plate B16 cells

1. Rapidly thaw a vial of cryopreserved B16 cells in a 37°C water bath until a small ice crystal is left in the vial.

   Prevent "heat shock" to tumor cells.

2. Transfer contents into a 15-ml tube containing 10 ml ice cold DMEM medium.

3. Centrifuge the cells for 10 min at 663 × *g* (1500 rpm, Sorvall RC4), 4°C.

   Cell pellet should appear light brown to black.

4. Decant supernatant and resuspend cells in 20 ml of ice-cold DMEM medium.

5. Plate in 162-cm^2 tissue culture–coated flask and grow in a 37°C, 5% CO_2 humidified incubator.

Maintain culture

6. Split 1:5 to 1:10 into a 162-cm^2 tissue culture flask when cells are 80% to 90% confluent (typically twice a week).

7. Use cells when in logarithmic growth phase (50% to 80%); this will yield 1–5 $\times$ 10^7 cells per 162 cm^2 flask.

ENUMERATING PULMONARY METASTASES IN MICE WITH B16 MELANOMA

SUPPORT PROTOCOL 2

Counting pulmonary nodules is facilitated by using a magnifying glass and lamp. Lungs with >250 pulmonary nodules are often scored as too numerous to count (TNTC) and assigned an arbitrary value of 250, since tumor nodules tend to fuse into continuous tumor sheets. Inclusion of such a nonlinear value necessitates a specific statistical test to determine significant differences between groups, i.e., the Kruskal-Wallis test, which can be found in every statistics textbook. Code the vials to enable subsequent counting in a blinded fashion.

Materials

B16-injected mice (see Basic Protocol 2)
Fekete's solution (see recipe)

Hardboard plate and pins
Sterile dissecting tools
Glass scintillation vials (PGC Scientifics)

Additional reagents and equipment for ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b) and euthanasia by CO_2 asphyxiation (*UNIT 1.8*; Donovan and Brown, 2006d)

Isolate lungs

1. Ear tag mice (*UNIT 1.5*; Donovan and Brown, 2006b), if necessary.

2. Euthanize mice by CO_2 asphyxiation (*UNIT 1.8*; Donovan and Brown, 2006d).

   Cervical dislocation tends to rupture cervical blood vessels, causing the chest cavity to fill with blood clots that complicate lung extraction and enumeration of tumor nodules. Euthanized mice may be stored for up to several days at 4°C without compromising tumor counts.

3. Pin mice face up on hardboard plate. Use push pins through hands and tail to stretch the body.

4. Open chest cavity with scissors, clipping a "V" from apex of sternum through ribs to upper left and right shoulder.

   Use small sharp scissors, taking care not to cut into lungs.

5. Use forceps to pull sternal plate towards and over head, exposing lungs and heart.

6. Using forceps, firmly grasp lung hilus directly above the heart and pull out lungs and heart as one unit.

   Ensure that no pulmonary lobes remain in chest cavity.

7. Rinse lungs in clean water to remove excess blood.

8. Place lungs in a labeled glass scintillation vial containing 5 ml Fekete's solution.

   Lungs should fix instantly and bleach to dull pink/white. B16 nodules will appear black, with occasional amelanotic, gray/white nodules.

9. After harvesting entirety of lungs, properly discard animal carcasses.

Enumerate nodules

10. Enumerate pulmonary nodules by a blinded investigator.

11. Using forceps, pry fixed lungs apart into individual lobes and enumerate tumor nodules on each lung. Also note total for all lobes.

    A lamp and magnifying glass are helpful.

12. Place lobes back in scintillation vial and store for future reference.

    The lobes may be stored indefinitely at room temperature in a tightly sealed vial.

13. Determine the average number of nodules for all mice within treatment groups.

SUPPORT PROTOCOL 3

BIOIMAGING OF LUCIFERASE-EXPRESSING TUMOR IN VIVO

This protocol requires the use of luciferase-expressing tumor and mice with light hair coat. Luciferase-expressing tumor can be generated by stable transfection of tumor cells with plasmid DNA encoding luciferase under the control of a mammalian constitutive promoter such as CMV promoter, or by transduction of tumor cells with retrovirus encoding the luciferase gene. Albino mice in various MHC backgrounds can be used if the hair coat color of original strain is dark or black. For example, in B16 tumor model, albino C57BL/6 mice are recommended.

Bioimaging can also be used to trace adoptively transferred T cells in vivo (Rabinovich et al., 2008). T cells are transduced with retrovirus expressing luciferase prior to adoptive transfer (see Support Protocol 10). Follow the same imaging procedures as described in this unit to trace the cells.

Materials

D-luciferin substrate (Caliper Life Sciences)
Mice with luciferase-expressing tumors (retroviral vectors for preparing these mice are available from Dr. W. Overwijk)
70% isopropyl alcohol prep (Webcol; Kendall Healthcare)
Dulbecco's PBS, without Ca^{++} and Mg^{++} (CMF-DPBS, Life Technologies)
Isoflurane

1-ml syringes
27-G, ½-in. needle
XGI-8 Gas Anesthesia System (Caliper Life Sciences)
IVIS 200 Pre-clinical In Vivo Imaging System (Caliper Life Sciences)
Living Image software (Caliper Life Sciences)

Inject luciferase substrate

1. Prepare D-luciferin substrate in CMF-DPBS at 15 mg/ml and fill a 1-ml syringe attached to a 27-G, ½-in. needle with this solution.

2. Restrain the luciferase-expressing-tumor-bearing mouse abdominal side up with one hand and use the other hand to wipe the lower left abdominal quadrant of mouse with an alcohol prep.

3. Keep mouse restrained and use the other hand to hold the syringe with the needle's bevel side facing up, slightly angled, and penetrate 4 to 5 mm into abdominal wall to inject intraperitoneally (also see *UNIT 1.6*; Donovan and Brown, 2006c) 100 μl substrate solution per 10 g of mouse body weight.

   A luciferin kinetic study should be performed for each animal model to determine peak signal time after luciferin administration. In general, mice should be imaged 5 to 15 min after the substrate injection.

Bioimage tumor

4. Place mice into a clear anesthesia box with 1% to 3% isoflurane.

   Because the peak of luciferin signal is time-sensitive, no more than five mice should be imaged together.

5. After mice are fully anesthetized, transfer the mice to the nose cones attached to the manifold in the imaging chamber.

   Practice the procedures to ensure that everything goes smoothly, to achieve the best image in an optimized time frame, especially when handle multiple mice at a time.

6. Close the door of the imaging chamber and acquire images according to manufacturer's instructions. Image both ventral and dorsal sides of the mice. After images are acquired, return the mice to their cages.

7. Designate regions of interest from displayed images and quantify as total photon counts or photon/second using Living Image software.

IMMUNIZATION WITH rVV, rFPV, OR rAd

SUPPORT PROTOCOL 4

The construction, amplification, and purification of recombinant vaccinia virus are described in detail in Earl et al. (1998). All viruses mentioned in this unit can be obtained through Dr. Nicholas Restifo (*restifo@nih.gov*) at the Surgery Branch, NCI, NIH. This protocol describes the use of recombinant vaccinia virus (rVV), recombinant fowlpox virus (rFPV), and recombinant adenovirus (rAd) in vivo. Make sure to contact the animal facility safety personnel to discuss safety and regulations for the use of viruses in a particular facility. Since rVV and rFPV are used identically, procedures are described for rVV only. The use of rVV and rFPV at a concentration $\geq 1 \times 10^9$ pfu/ml is recommended, since use of crude, unpurified, or low-titered preparations can result in the injection of relatively large and potentially lethal quantities of cellular debris and contaminants originating from the amplification cell line.

Typical doses that induce reliable immunization upon intravenous injection are 1×10^7 plaque forming units (pfu) of rVV or rFPV, and 1×10^9 pfu of rAd. These immunizations have induced CTL and antibody responses to foreign antigens such as β-galactosidase, chicken ovalbumin, and influenza nuclear protein, as well as the "self" proteins gp100, tyrosinase, and TRP-2 (Overwijk et al., 1997, 1998; Bronte et al., 2000; Colella et al., 2000). Two immunizations with 1×10^7 pfu rVVmTRP-1 will induce mTRP-1 specific antibodies, vitiligo, and tumor protection in essentially 100% of mice (Overwijk et al., 1999a).

If necessary, mice can be boosted with virus or another immunogen, such as plasmid DNA, to enhance the immune response. Vaccine vectors used for boosting should preferably be different from those used for primary immunization, to prevent rapid clearance of the vector by the vector-specific immune response induced during primary immunization. Therefore, using rVV followed by rFPV or rAd is more effective than rVV followed by rVV (Irvine et al., 1997).

CTL responses peak around 6 days after primary immunization or 4 days after booster immunization (Irvine et al., 1997). Spleens or serum can be harvested 2 to 3 weeks after the last immunization. This is also the optimal time to challenge mice with B16; CTL and antibody levels tend to gradually decrease with time.

Materials

rVV or rFPV stock at a titer $> 1 \times 10^9$ pfu/ml, or rAD at a titer $> 1 \times 10^{10}$ pfu/ml
PBS (Life Technologies), ice cold
6- to 12-week old female C57BL/6 mice

Cup sonicator filled with ice water
Heat lamp
1-ml disposable syringes and 27-G, ½-in. needles

Additional reagents and equipment for mouse restraint (*UNIT 1.3*; Donovan and Brown, 2006a), ear tagging (*UNIT 1.5*; Donovan and Brown, 2006b), and intravenous injection of mice (*UNIT 1.6*; Donovan and Brown, 2006c)

Prepare virus

1. Thaw rVV, rFPV, or rAd viral stock in 37°C water bath.

   From this point on, all procedures are performed on ice.

2. *Optional:* Sonicate virus stock (in case of rVV or rFPV) using an ice-water-filled cup sonicator at full power for 30 sec.

   This breaks up clumps formed during freezing. Wear ear protection.

3. Dilute virus in ice-cold PBS to desired concentration.

   Typically 2×10^7 pfu/ml for rVV or 2×10^9 pfu/ml for rAd.

   Viral stocks can be refrozen. rVV and rFPV are quite stable and can be freeze-thawed several times without major reduction in titers. rAd is less stable depending on its diluents and should preferably be aliquotted after purification so that no refreezing is necessary.

Immunize mice

4. Relocate work area to the facility where the 6- to 12-week old female C57BL/6 mice are kept, while keeping virus on ice.

5. Warm mice by placing under heat lamp for 2 to 3 min, until tail veins are dilated.

6. Immobilize mice using a restraint that allows tail to protrude (*UNIT 1.3*; Donovan and Brown, 2006a). Inject 0.5 ml virus intravenously in tail vein (*UNIT 1.6*; Donovan and Brown, 2006c) using a 1-ml syringe and 27-G, ½-in. needle.

Randomize mice

7. If mice have not been pretreated, randomize mice after they have been injected. Ear tag mice (*UNIT 1.5*; Donovan and Brown, 2006b) to blind the experiment.

SUPPORT PROTOCOL 5

DETECTING ANTIBODIES AGAINST B16 MELANOCYTE DIFFERENTIATION ANTIGENS (MDA) BY ELISA

It is recommended that a large batch of B16 lysate be made and frozen it in 5-ml aliquots, since there can be considerable variation between batches. B16 lysate loses reactivity with increasing freeze-thaw cycles; for this reason, use a fresh aliquot for each experiment.

It is of critical importance to include a lysate made from cells that do not contain the antigen(s) of interest as a negative control, along with the B16 lysate to control for nonspecific reactions.

Instead of B16 melanoma, non-melanoma cells transfected with plasmid DNA or infected with viral vectors encoding MDA can be used. However, if antibody responses were induced using recombinant viruses, the resulting high titers of antiviral antibodies will typically obscure most specific antibody responses to the transgene if virus-infected cells are used as the source of antigen for ELISA. This protocol is less sensitive for detecting antibodies than protocols that use immunoprecipitated MDA from lysates of metabolically labeled (^{35}S-methionine) B16 cells (Naftzger et al., 1996). However, it is faster and does not require the use of radioactivity.

Typical dilutions of sera are 1:10, 1:50, 1:250, 1: 1,250, 1:6,250, and 1:31,250. Dilutions are made in PBS with 1% BSA.

Materials

B16 culture
PBS (Life Technologies), ice cold
Liquid N_2
1% and 5% (w/v) BSA in PBS (Life Technologies)
Sera from immunized mice
HRP-conjugated anti-mouse Ig (Amersham Pharmacia Biotech)
TMB substrate (Life Technologies)
4 M H_2SO_4

Disposable cell scraper
Refrigerated centrifuge
Vacuum aspirator
Freeze-resistant tubes
PVC microtiter plates (Dynex Technologies)
SkanWasher 300 (Skatron Instruments)
ELISA reader

Additional reagents and equipment for counting viable cells (*APPENDIX 3A & 3B;* Strober, 1997a, b)

Prepare B16 cell lysate

1. Ensure that B16 cells are 50% to 80% confluent.
2. Decant medium. With a cell scraper, scrape B16 cells from culture flask and resuspend in 10 ml ice-cold PBS. Pipet vigorously to obtain a single-cell suspension.

   If necessary, pass through a cell strainer.

   Do not use trypsin, since it may cause proteolysis of the MDA of interest.
3. Count cells (*APPENDIX 3A & 3B*; Strober, 1997a,b). Centrifuge the cells for 5 min at 663 × *g*, 4°C.
4. Carefully remove all supernatant using vacuum aspiration. Resuspend cells at 2 × 10^5 cells/ml in PBS, transfer to a freeze-resistant tube, and lyse using three freeze/thaw-cycles.

   Use freeze-resistant tubes; alternate between liquid nitrogen and 37°C water bath.

Coat and block plates

5. Plate 50 μl/well of lysate (equivalent to 1×10^4 cells) into PVC microtiter plates. Dry overnight at 37°C.
6. Block with 100 μl 5% BSA in PBS per well for 1 hr at 37°C. After 1 hr, discard blocking buffer from ELISA plates.

   Remove remaining buffer by clapping inverted plate on a hard surface.

Probe wells with serum samples

7. Add 50 μl mouse sera diluted in PBS with 1% BSA per well and incubate for 2 hr at 37°C.

   Typical dilutions of sera are 1:10, 1:50, 1:250, 1: 1,250, 1:6,250, and 1:31,250.

8. Wash wells three times with 1% BSA in PBS, room temperature.
9. Incubate with HRP-conjugated anti-mouse Ig at 1:4000 dilution in 1% BSA in PBS for 1 hr at 4°C.
10. Wash three times with 1% BSA in PBS, room temperature, using a SkanWasher 300 or by manually aspirating all liquid in the wells and adding 300 μl/well of 1% BSA/PBS. Repeat aspiration and addition four times.

Develop enzymatic reactions

11. Add 50 μl of TMB substrate per well and incubate for 10 min at room temperature.
12. Stop reaction by adding 50 μl 4 M H_2SO_4 per well.
13. Read plate at 492 nm in an ELISA reader.

SUPPORT PROTOCOL 6

GENERATING B16 MELANOMA-SPECIFIC T CELL CULTURES

This protocol can be used to test the degree of immunization against specific antigens upon vaccination. It assumes that an MHC Class I restricted peptide epitope has been identified for in vitro restimulation and testing. Alternatively, irradiated B16 tumor cells can be used as stimulators; however, in the authors' experience this often results in outgrowth of TRP-2 reactive CTL, even when stimulating splenocytes from naive mice (Bloom et al., 1997; Zeh III et al., 1999). This protocol has been used to determine efficacy of immunization with recombinant viruses (i.e., rVV, rFPV, rAd; see Support Protocol 4), whole tumor cells (see Basic Protocol 4), recombinant DNA, whole protein, and synthetic peptides. T cell cultures are initiated 2 to 3 weeks after vaccination.

Materials

Immunized mice, at least 2 mice per group (see Basic Protocols 3 and 4)
HBSS (Life Technologies), ice cold
ACK Lysing buffer (Life Technologies)
Complete medium (CM;see recipe), ice cold
Peptide stocks: 10 mg/ml in DMSO

Sterile dissecting instruments
70% isopropyl alcohol in spray bottle
100-μm sterile disposable cell strainers (BD Biosciences)
3-ml syringe
50-ml conical tubes (e.g., BD Falcon)
Refrigerated centrifuge (such as Sorvall RC4)
25-cm^2 tissue culture flasks

Additional reagents and equipment for euthanasia of mice (*UNIT 1.8*; Donovan and Brown, 2006d) and counting viable cells (*APPENDIX 3A & 3B*; Strober, 1997a,b)

Prepare single-cell suspension from spleen

1. Euthanize immunized mice using cervical dislocation (*UNIT 1.8*; Donovan and Brown, 2006d).
2. In a biosafety cabinet, using sterile instruments, remove spleens, place into a 50-ml conical tube (one spleen per tube) with 5 ml ice-cold HBSS, and place on ice.

   Spleens are obtained through incision in the left flank after wetting of fur with 70% isopropyl alcohol.

3. Insert a 100-μm disposable cell strainer into a 50-ml conical tube. Transfer spleens to the strainer and mash spleens thoroughly with the sterile end of a plunger from a 3-ml syringe.
4. Rinse the strainer with 10 ml of cold HBSS and discard the cell strainer.
5. Close the cap and centrifuge the cell suspension for 5 min at 779 × *g* (1800 rpm, Sorvall RC4), 4°C.
6. Discard the supernatant and resuspend the cell pellet with 5 ml of ACK Lysing buffer. Incubate at room temperature for 5 min.
7. Add 45 ml of cold HBSS to the tube and centrifuge the cell suspension for 5 min at 600 × *g*, 4°C
8. Add 10 ml of CM to the cell pellet and pipet vigorously up and down to obtain a single-cell suspension.

   UNIT 3.1 (Kruisbeek, 2000) contains additional details on preparation of spleen cell suspensions.

9. Count cells (*APPENDIX 3A & 3B*; Strober, 1997a, b).
10. Divide cells into groups—e.g., specific peptide(s), control peptide(s)—and culture 2×10^6 cells per ml CM in a 25-cm^2 tissue culture flasks.

    Typically 10 to 30 ml CM per flask is used. Alternatively, cells can be cultured at 3–5 × 10^6 cells/well in 2 ml CM/well in 24-well plates. No rIL-2 is added.

Stimulate cultures

11. Add 1 to 10 μM final concentration of the appropriate stimulator peptide.

    This roughly equals 1 to 10 μg/ml for 9-mer peptides.

12. Incubate flasks horizontally. Culture for 7 to 10 days at 37°C before testing or restimulation.

    Slightly unscrew caps or use vented caps to allow gas exchange.

FUNCTIONAL TESTING OF B16 MELANOMA–SPECIFIC T CELLS

SUPPORT PROTOCOL 7

When testing CTL lines, especially new secondary cultures, it is critical to include two types of negative controls. First, a CTL culture or line that is not antigen-specific must be included. Some CTL lines, and in particular secondary cultures, often display nonspecific recognition of tumor cells, especially B16, which can be a good stimulator of some T cell lines with unrelated specificity. The best control for this is CTL generated from the same splenocytes (i.e., same mice) as the CTL cultures of interest, except using a 7-day culture of the splenocytes with either a different peptide or no peptide. The recognition of B16 by this nonspecific CTL culture should be carefully compared to the recognition of B16

by the CTL culture of interest. This controls for the ability of B16 to nonspecifically stimulate cytokine secretion by any CTL culture it is incubated with; this nonspecific secretion can be quite pronounced.

The second control that should be included is one or more nonspecific target cells, i.e., any tumor cell line that is antigen negative and is $H\text{-}2^b$. Examples are sarcomas from the MCA series, EL-4 thymoma, or MC 38 colon carcinoma. This controls for the nonspecific reactivity of the CTL cultures of interest; some CTL cultures can "recognize" essentially any tumor with which they are incubated.

It is important to keep vials of peptide stock solution physically separated. These peptides are highly stable and, due to their high concentrations, the smallest peptide cross-contamination can result in erratic "recognition" upon use in CTL assays. Ethanol does not destroy peptides, and any peptide clinging to the outside of the vial, gloves, etc., can easily cross-contaminate another vial.

Materials

B16-specific CTL bulk culture (see Support Protocol 6)
Culture medium (CM; see recipe)
Target cells, including B16 and control tumor cell lines
10 mg/ml peptide stock in DMSO
IFN-γ ELISA kit

Sterile reagent basins
50-ml conical tubes
Refrigerated centrifuge (such as Sorvall RC4)
96-well round-bottom plates

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997a) and determining viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b)

Harvest CTL cultures

1. Suspend B16-specific CTL from wells or flasks by vigorously pipetting medium up and down. Collect all cells into a reagent basin and transfer the cell suspension to 50-ml conical tubes.
2. Centrifuge cell suspension for 5 min at 779 × *g* (1800 rpm in a Sorvall RC4), room temperature.
3. Decant supernatant and resuspend cell pellet in CM at a final concentration of 1–5 × 10^6 cells/ml.
4. Count cells using trypan blue to exclude dead cells (*APPENDIX 3A & 3B*; Strober, 1997a,b. Dilute cells to 5 × 10^5 cells/ml in CM.

   There will be quite a few dead cells.

5. Plate 100 μl cells/well (5 × 10^4 cells) in round-bottom 96 well plate.

   If signal is expected to be weak, use 1 × 10^5 cells/well.

Prepare targets

6. Prepare target cells at 5 × 10^5 cells/ml.
7. For peptide-pulsed targets, incubate target cells with 1 to 10 μM peptide in CM at 37°C for 2 hr. Wash in CM, then centrifuge for 5 min at 779 × *g* (1800 rpm in a Sorvall RC4), 4°C, to remove free peptide. Repeat twice, for a total of three washes.

Alternatively add 100 μl peptide (2 to 20 μM in CM) directly to CTL. The CTL will present peptide to each other on MHC Class I and subsequently kill each other, but not before secreting high quantities of IFN-γ. Adding peptide directly tends to yield a more sensitive reading than using peptide-pulsed tumor targets; it also eliminates possible background recognition of the tumor target itself.

Co-culture CTL and targets

8. Add 100 μl of target cells (5×10^4 cells) to each well containing T cells.
9. Incubate co-culture plates overnight at 37°C.

In cases of established CTL lines, lysis of targets can sometimes be scored visually, which can be useful when screening large numbers of cultures; however IFN-γ ELISA is a more sensitive assay.

Test supernatant

10. Harvest supernatants from wells.

Most ELISA kits use 50 μl supernatant per sample; freeze remainder as backup.

If necessary, supernatants can be stored up to several months at –20°C for later use.

11. Perform IFN-γ ELISA on supernatants according to instructions provided with the kit.

Analyze results

12. Compare cytokine production by CTL cultures of interest when co-cultured with B16 to that of control tumors; also compare specific peptide to nonspecific peptide.

Values for nonspecific peptide should be similar to values for CTL in CM alone; if not, use a different control peptide, since cross-reactivity can occur reproducibly between non-sequence-related peptides. Also, ensure that nonspecific CTL lines are not stimulated by B16 to produce cytokines.

To establish CTL lines from any short-term cultures, purify cells by Ficoll gradient and restimulate CTL on day 7 to 10 after start of secondary culture, as described in Support Protocol 6.

MAINTAINING B16-SPECIFIC CTL CULTURES

SUPPORT PROTOCOL 8

Growing CTL takes practice, and may be somewhat more difficult for "self" antigens like gp100 than for "non-self" antigens such as β-galactosidase or ovalbumin. The following protocol is one of many variants; each laboratory tends to use a different modified protocol. Experimenting is key, as is daily observation of cultures. Murine CTL are very unforgiving; neglect—sometimes just for 1 day—can result in rapid death or irreversible conversion from spindle-shaped, specific CTL into large, round, granular cells that grow faster but display high background reactivity and ultimately lose all specificity. Keeping cultures "clean," i.e., free of too much cell debris, tends to suppress this phenomenon, as does maintaining the CTL in relatively low IL-2 concentrations (e.g., between 30 and 60 IU/ml rhIL-2). As an alternative strategy, some investigators prefer not to add IL-2 for the first 1 or 2 days after restimulation.

Despite the establishment of international units (IU) for biological response modifiers like IL-2, IU values differ among manufacturers. The guidelines mentioned here are for human recombinant IL-2 (Proleukin or Aldesleukin); some experimenting with concentrations may be required when using a different source. RBC lysis of feeder splenocytes is not required for restimulation, but does result in less debris in CTL cultures.

Restimulate CTL lines every 7 to 14 days. Cultures will need the most attention during the first 5 days after restimulation; restimulating on Mondays may help accommodate this requirement. Observe cultures daily; initially (i.e., day 1 to 3 after restimulation) it may appear as if no CTL remain; however, they will suddenly re-emerge as feeder splenocytes disappear. At this point (i.e., day 3 to 7), CTL numbers can double every 12 to 24 hr, requiring close observation to prevent overcrowding of the wells. As soon as CTL begin to reach confluency, split 1:2 to 1:4 into new wells and fill wells to 2 ml with CM/IL-2. Regardless of growth, replace 1 ml of medium with CM/IL-2 every other day to ensure continuous presence of IL-2. Proliferation will typically slow down after day 5 to 10. CTL will then "calm down"; they appear less bloated, more spindle-shaped, and somewhat smaller. This is the best time for use in CTL recognition assays, i.e., between day 0 and 3, CTL tend to display high background reactivity. CTL can remain for up to 2 weeks at stable or slowly decreasing numbers in CM/IL-2 without restimulation, as long as IL-2 is regularly provided. The cells remain highly specific throughout this time, making this a useful strategy to always have CTL available for CTL assays.

B16 itself can be used to restimulate B16-specific CTL cultures. It is important to heavily irradiate B16, since it is very radiation resistant and will overgrow CTL cultures if given less than 40,000 rad. Even after this large dose, B16 can persist for weeks and continue to rapidly acidify culture medium (overnight) if not killed by CTL. It is therefore important not to exceed 1×10^5 irradiated B16 cells/well (with 3×10^6 cells/well unpulsed feeder splenocytes in 24-well plate), especially when restimulating poorly lytic CTL cultures. Peptide-pulsed, lightly irradiated splenocytes tend to give greater stimulation and expansion and disappear within days.

Materials

B16-specfic CTL (see Support Protocol 6)
Complete medium (CM; see recipe), ice cold
Recombinant human IL-2
6- to 12-week old female C57BL/6 mice
10 mg/ml peptide in DMSO

24-well plates
γ irradiator
50-ml conical tubes

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3A*; Strober, 1997a), determination of viable cells by trypan blue exclusion (*APPENDIX 3B*; Strober, 1997b), isolation of splenocytes (Support Protocol 6, steps 1 to 8), and CO_2 euthanasia (*UNIT 1.8*; Donovan and Brown, 2006d)

Harvest CTL

1. Suspend CTL from wells or flasks by vigorously pipetting medium up and down.

   If cultures contain a large amount of debris, purify by Ficoll gradient. To do this, centrifuge cells 5 min at 600 × g, room temperature. Count cells (APPENDIX 3A & 3B; Strober, 1997a,b) and resuspend to roughly 1×10^7/ml in CM. It is important to keep the cells at room temperature. Carefully underlay 5 ml of Lympholyte-M (Cedarlane, room temperature) and centrifuge according to manufacturer's recommendations.

2. Resuspend CTL at 1×10^6 cells/ml in CM containing 60 IU/ml IL-2.

3. Plate 1 ml CTL per well in 24-well plate.

Prepare stimulator cells

4. Isolate splenocytes from mice according to steps 1 to 8 of Support Protocol 6. Adjust the cell concentration to 1×10^7 cells/ml.
5. Incubate splenocytes with 1 to 10 μM peptide for 2 hr at 37°C.

   For peptides with 9 amino acids, 1 μM is equivalent to 1 μg/ml.
6. Irradiate splenocytes at 30 Gy.
7. Wash the irradiated splenocytes three times in 50 ml of CM by centrifugation for 5 min at 779 × *g* (1800 rpm Sorvall RC4), 4°C.
8. Decant supernatant and resuspend cell pellet in CM/IL-2 to a final concentration of 3×10^6 cells/ml.

Add stimulators to CTL

9. Add 1 ml peptide-pulsed irradiated stimulator splenocytes to plated CTL (from step 8)

   Alternatively, use 3×10^6 unpulsed splenocytes and add 1×10^5 B16 (400 Gy irradiated)
10. Culture cells at 37°C, overnight.

Observe daily and split when necessary

11. The following day, replace 1 ml of the medium with 1 ml of fresh CM containing 60 IU/ml IL-2.

    Observe daily under microscope; replace 1 ml medium every other day with CM/IL-2.

    When CTL become confluent, split 1:2 to 1:5.

CULTURING PMEL-1 TRANSGENIC T CELLS

SUPPORT PROTOCOL 9

Pmel-1 transgenic T cells recognize mouse MDA $gp100_{25\text{-}33}$ epitope in the context of *H-2K^b*. Pmel-1 transgenic mice were developed in the authors' laboratory more than a decade ago and have become useful tools to study the biology of adoptive transfer in tumor treatment model (Overwijk et al., 2003). Although naive lymphocytes from Pmel-1 transgenic mice can be used in adoptive transfer, it is more common to use ex vivo–expanded Pmel-1 cells. Between 20% and 30% of freshly isolated lymphocytes from Pmel-1 transgenic mice are $CD8^+$ T cells, and more than 90% of those $CD8^+$ T cells express TCR Vβ13. After 7 days of ex vivo culture with $gp100_{25\text{-}33}$ peptide and rhIL-2, more than 90% of cells in the culture are $CD8^+V\beta13^+$ T cells. In general, a 100- to 500-fold increase in number of Pmel-1 cells can be achieved using the culture method described in this unit. Although the most common source of lymphocytes is spleen, lymph nodes and peripheral blood can also be used to culture Pmel-1 T cells.

Materials

6- to 8-week old Pmel-1 transgenic mice (Jackson Laboratory)
Complete medium (CM; see recipe)
Recombinant human IL-2
10 mg/ml $gp100_{25\text{-}33}$ peptide stock (CPC Scientific, cat. no. 834139) in DMSO
24-well plates
Multi-channel pipettor with 1200-μl pipet tips

Additional reagents and equipment for isolation of splenocytes (Support Protocol 6, steps 1 to 8)

Isolate splenocytes

1. Isolate splenocytes from Pmel-1 transgenic mice according to steps 1 to 8 of Support Protocol 6. Adjust the cell concentration to 1.5 to 2 $\times$ 10^6 cells/ml in CM containing 60 IU/ml IL-2

   A normal-size spleen usually generates 50 ml of cell suspension at the required concentration. Although freshly isolated splenocytes can be cryopreserved for future use, ex vivo expansion of Pmel-1 cells requires additional irradiated feeder cells if frozen splenocytes are used.

Culture Pmel-1 cells

2. Add peptide stock to the cell suspension at the final concentration of 1 μg/ml. Mix well by inverting the tube repeatedly.
3. Add 2 ml of the cell suspension into a well of 24-well plate and incubate for 2 days in a 37°C, 5% CO_2 humidified incubator.

   In general, these cells do not need to be split during the first 48 hr of peptide stimulation. Close contact of cells during this period facilitates cell proliferation. After 24 hr in culture, look for signs of activated T cells, i.e., cell colony formation and elongated or "tennis racket" shaped cells, especially around the edge of the well (Fig. 20.1.1).

4. Split cells every day after 2 days of culture using a multi-channel pipettor with 1200-μl pipet tips.

   Fresh CM containing 60 IU/ml IL-2 medium is added to the wells—no additional peptide is needed.

   Cells need to be monitored daily. During the rapid proliferation phase of Pmel-1 T cells, a high density of cells is detrimental to the growth. Maintain 50% to 80% confluence for optimal cell growth.

5. On day 7, harvest Pmel-1 cells from 24-well plate using a multi-channel pipettor with 1200-μl pipet tips.

   Cells can be frozen or used immediately in an adoptive transfer experiment as described in Basic Protocol 6.

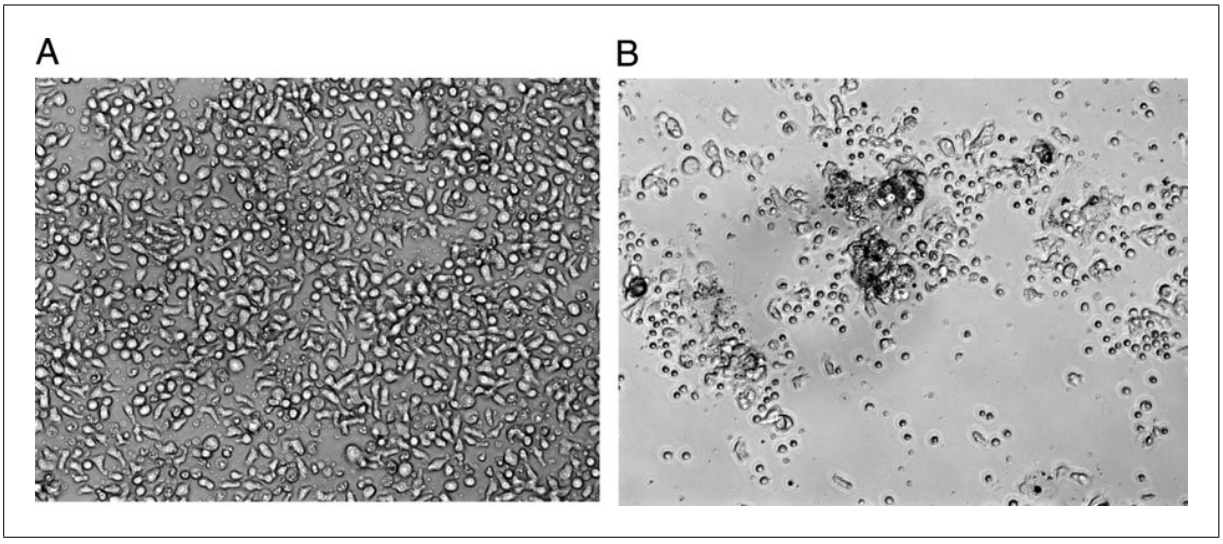

Figure 20.1.1 Fresh Pmel-1 splenocytes after 7 days of culture in 1 μg/ml $gp100_{25–33}$ peptide and 60 IU/ml IL-2 CM (activated, **A**) or in CM (non-activated, **B**).

A typical 7-day culture of one Pmel-1 spleen can yield 16 to 32 24-well plates with 2–5 × 10^7 cells/plate. Frozen Pmel-1 cells at day 7 of culture can be recovered in CM/IL-2 overnight at 37°C in a 5%CO_2 humidified incubator and are ready to use for adoptive transfer. Some investigators use as early as day-4 culture for adoptive transfer.

GENE MODIFICATION OF MOUSE T CELLS BY γ-RETROVIRAL TRANSDUCTION

SUPPORT PROTOCOL 10

In the past, mouse T cells were hard to transduce with retrovirus compared to human T cells. With the improvement of viral constructs and optimization of transduction conditions, gene expression is greatly enhanced upon retroviral transduction. In many cases, the efficiency can reach as high as 90%. Therefore, it has become a very useful tool to modify T cells for adoptive transfer.

The viral construct is critical to the success of gene engineering in mouse T cells. In the authors' laboratory, one of the optimized vectors is MSGV1 (MSCV-based splice-gag vector). MSGV1 is derived from murine stem cell virus (MSCV) long terminal repeat (LTR) and contains an extended gag region and env splice site (Hughes et al., 2005). When more than one gene product needs to be co-expressed in a same cell, such as α and β chain of T cell receptors, the two cDNA sequences are linked by either a sequence encoding the foot-and-mouth disease picornavirus 2A ribosomal skip peptide or an internal ribosomal entry site, IRES.

Freshly prepared ecotropic γ-retrovirus is a more efficient transducer of mouse T cells compared to frozen viral supernatant. Making a γ-retroviral producing line for mouse T cell transduction can be time intensive. For most exploratory experiments, it is not worth the effort. Therefore, many investigators use transiently produced retroviral supernatant for mouse experiments. However, to ensure reproducible results, it is very important to keep the reagents (such as plasmid DNA, Lipofectamine, and RetroNectin) and experimental procedures consistent.

Many investigators have found use of RetroNectin during transduction an irreplaceable element; others have successfully used protamine sulfate as a cost-cutting and time-saving substitute. In this unit, both methods are described.

Materials

Platinum-E retroviral packaging cell line ecotropic, (Plat-E, Cell Biolabs Inc.) or other 293 based retroviral packaging cell line such as 293-GP
DMEM medium, with and without antibiotics (see recipe)
Opti-MEM medium (Life Technologies)
Retroviral vector (see protocol introduction)
Envelope vector such as pCL-eco (Addgene, cat. no. 12371) for ecotropic retroviral production
Lipofectamine 2000 (Life Technologies)
Complete medium (see recipe)
Recombinant human IL-2
Concanavalin A (ConA, Sigma)
Recombinant murine IL-7
Phosphate-buffered saline (PBS, Life Technologies), sterile
RetroNectin (Clontech), prepared at 1 mg/ml in sterile water according to the manufacturer's instruction
Protamine sulfate (optional; as an alternative to RetroNectin)
2% (w/v) bovine serum albumin in PBS, sterile filtered

100-mm poly-D-lysine coated tissue culture dish (BioCoat; Corning, cat. no. 356469)
3-ml polypropylene tubes, sterile
24-well tissue culture plates
24-well non–tissue culture treated plates
20-ml syringes
0.45-μm filters
15-ml conical centrifuge tubes
Refrigerated centrifuge with biohazard-protection-covered rotor buckets adapted for tissue culture plates.

Additional reagents and equipment for counting cells viable cells in a hemacytometer by trypan blue exclusion (*APPENDIX 3A & 3B*; Strober, 1997a,b), and harvest of splenocytes (Support Protocol 4, steps 1 to 8)

Transfect γ-retroviral packing cells

All cell culture procedures should be performed in a biological safety cabinet and follow proper sterile and Biosafety level 2/3 techniques.

1. *Day 0:* Plate Plat-E or 293 GP cells at 6.0×10^6 in 10 ml of DMEM medium in 100-mm poly-D-lysine coated dishes

   Cell density should reach around 80% confluence on the day of transfection.

2. *Day 2:* Change medium to 10 ml of DMEM medium without antibiotics. Place the cells back in the incubator and proceed to preparation of transfection reagents.

   Transfection is usually done in the morning because the medium need to be changed in 6 to 8 hr.

3. To a sterile 3-ml polypropylene tube, labeled "A," add 1.5 ml of Opti-MEM, 9.0 μg of retroviral vector, and 6 μg of Envelope vector. Mix well by pipetting up and down. To another sterile 3-ml polypropylene tube, labeled "B," add 1.5 ml of Opti-MEM and 60 μl of Lipofectamine 2000 and gently mix well.

4. Using a pipet, gently mix solution from tube B into tube A. Do not vortex. Incubate the DNA/lipid complex mixture at room temperature for 20 min.

5. Add 3 ml DNA/lipid complex mix dropwise to the packaging cells in a 100-mm dish (from step 1; total, 13 ml medium). Incubate the cells for 6 to 8 hr at 37°C.

6. Replace the medium with 10 ml DMEM medium (with antibiotics) and incubate at 37°C for 36 to 48 hr.

Activate T cells

7. *Day 2:* Harvest splenocytes from mice and prepare single-cell suspension according to steps 1 to 8 of Support Protocol 4. Adjust cell concentration to 2×10^6 cells/ml in CM containing 60 IU/ml recombinant human IL-2.

8. Add 2.5 g/ml ConA and 1 ng/ml IL-7 into the cell suspension and mix well by pipetting up and down.

9. Add 2 ml of cell suspension per well of a 24-well non-tissue-culture-treated plate. Incubate at 37°C for 48 hr in a 5% CO_2 humidified incubator.

Coat plates with RetroNectin (skip if use protamine sulfate)

10. *Day 3:* Prepare 50 μg/ml RetroNectin in sterile PBS and add 0.5 ml per well of 24-well non-tissue culture–treated plates. Incubate the coated plates overnight at 4°C.

11. *Day 4:* Wash the coated plates twice, each time with 2 ml of sterile PBS, then block the plates with 1 ml/well of sterile PBS with 2% BSA at room temperature for 30 min. Wash twice with sterile PBS. Place the plates in the biosafety hood and proceed to the next step.

Harvest retroviral supernatant

12. *Day 4:* Collect supernatant from transfected Plat-E cells using 20-ml syringe and push through a 0.45 μM filter attached to the syringe into a sterile 15-ml tube.

    There is about 13 ml of supernatant from each 100 mm dish, which is enough for one 24-well plate.

Transduce activated T cells

The following steps are to be performed on day 4.

13. Dilute retroviral supernatant with an equal volume of CM and add 1 ml/well to the blocked RetroNectin-coated plates from step 11 (at room temperature). Place the plate in a covered plate rotor and centrifuge for 2 hr at 2000 × *g*, room temperature.

    If biohazard-covered rotor is not available, the plates can be wrapped with parafilm and place in a plastic bag during the centrifugation to prevent contamination. This step is not necessary if using protamine sulfate.

14. During centrifugation, harvest activated T cells and resuspend T cells at 1×10^6 cells/ml in CM containing 60 IU/ml IL-2.

    If using RetroNectin, go to step 16.

15. Add 1.5 μg/ml protamine sulfate to activated T cells (from step 9) and plate 1 ml/well (1×10^6 cells/well) of cell suspension into a 24-well tissue culture plate. To each well, add 0.5 ml of retroviral supernatant (from step 12) and centrifuge the plate in biohazard-covered rotor for 1.5 hr at 1000 × *g*, room temperature, followed by incubation at 37°C in a 5%CO_2 humidified incubator.

    Proceed to step 18.

16. After centrifugation of retroviral supernatant (from step 12) onto the RetroNectin-coated plates, remove the supernatant. Do not discard the supernatant. Add 1 ml/well (1×10^6 cells/well) of activated T cell suspension (from step 9) into each well and centrifuge the plate in a biohazard- covered plate rotor for 10 min at 1000 × *g*, room temperate. Incubate the plates at 37°C in a 5% CO_2 humidified incubator.

    Transduction efficiency can be enhanced by a second round of transduction on the following day. After being removed from the RetroNectin-coated plates, the retroviral supernatant can be added to a new RetroNectin-coated plate and the centrifugation in step 13 repeated. The plate can then be stored in 4°C and used the next day, following step 16.

17. If using RetroNectin, 24 hr after the last transduction, transfer T cells into a 24-well tissue culture plate and continue incubation at 37°C.

18. Split cells in CM containing 60 IU/ml IL-2 daily 48 hr after transduction.

    Gene expression can be assessed 72 hr after last transduction. T cells can be used for adoptive cell transfer 5 to 7 days post transduction.

    Cell viability is generally 80% to 90% within 7 days after transduction and is reduced after 7 days. In some cases, when the transduction efficiency is very high (>90%), the cell viability can decline within 7 days after transduction. Washing the cells with CM containing 60 IU/ml IL-2 during the split may help the cell viability.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps, see ***APPENDIX 2A****; for suppliers, see* ***APPENDIX 5****.*

Complete medium (CM)

10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)
1 mM sodium pyruvate
2 mM L-glutamine or Glutamax
100 μM nonessential amino acids
100 U/ml penicillin
100 μg/ml streptomycin
50 μg/ml gentamicin
0.05 mM 2-mercaptoethanol
Bring up to 500 ml with RPMI 1640 (Life Technologies)

For CM/IL-2: add 30 to 60 IU/m recombinant human IL-2, store up to 2 weeks in the dark at 4°C.

Il-2 is quite stable in CM (weeks).

DMEM medium

10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)
1 mM sodium pyruvate
2 mM L-glutamine or Glutamax
100 μM nonessential amino acids
100 U/ml penicillin
100 μg/ml streptomycin
50 μg/ml gentamicin
Bring up to 500 ml with DMEM (Life Technologies)
Store up to 2 weeks at 4ºC
For DMEM without antibiotics:
10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)
1 mM sodium pyruvate
2 mM L-glutamine or Glutamax
100 μM nonessential amino acids
Bring up to 500 ml with DMEM (Life Technologies)
Store up to 2 weeks at 4ºC

FACS buffer

2.5 g bovine serum albumin (BSA)
0.02 g sodium azide
Dissolve in 500 ml of PBS (Life Technologies)
Store up to 6 months at 4ºC

Fekete's solution

For 1 liter:
580 ml 95% ethanol
200 ml H_2O
80 ml 37% formaldehyde solution
40 ml glacial acetic acid
Stable at room temperature indefinitely

CAUTION: Potential irritant.

COMMENTARY

Background Information

B16 melanoma, a spontaneous melanoma derived from a C57BL/6 mouse, has been used in many pre-clinical studies to model human cancer immunotherapy, including the authors' own research. There are many characteristics of this tumor line that have made it an attractive model.

As a model for human tumors, it is important to note similarities and differences between B16 and human melanomas (see Table 20.1.3). Several variants were isolated by Dr. Isaiah J. Fidler in the mid-1970s, the main differences between sub- lines being their metastatic potential and susceptibility to immune destruction (Fidler et al., 1976, Fidler and Bucana, 1977). The most commonly used variant is B16.F10, which is highly aggressive and will metastasize from a primary subcutaneous site to the lungs, as well as colonize lungs upon intravenous (i.v.) injection. The parental line is named B16.F1; other variants are B16.F0, a poorly metastatic variant, and B16.BL6, which has a high degree of metastasis and is subject to intermediate colonization of lungs (Hart, 1979). Most laboratories use B16.F10 for in vivo experiments.

B16 melanoma is one of the very few pigmented melanoma lines available for use in mice, although, recently, transgenic models have been developed in which the reliable incidence of melanoma allows the establishment of new lines (Chin et al., 1997; Otsuka et al., 1998; Zhu et al., 1998). In addition, a recent report described a retrovirus derived from B16 itself that can transform normal, cultured mouse melanocytes to establish melanoma tumor lines that grow in normal, immunocompetent mice (Li et al., 1998). Interestingly, these tumor cell lines, like B16, express low levels of MHC Class I compared to other C57BL/6 derived tumors such as the methylcholanthrene-induced sarcomas (MCA series) and other commonly used cells such as EL-4 thymoma or normal splenocytes. This low expression of MHC Class I appears to be a feature of normal C57BL/6 mouse melanocytes that is retained after transformation to melanoma (Li et al., 1998; Xu et al., 1998).

B16 melanoma has classically been described as a non- or low-immunogenic tumor. One reason for this designation is the difficulty in inducing protection against B16 challenge by injection of irradiated B16, regardless of the addition of Bacillus Calmette Guérin (BCG) or *Corynebacterium parvum* (*C. parvum*), a strategy that can induce reliable protection in many other tumor models. B16 is also relatively resistant to treatment with high-dose Il-2, a therapy that can essentially cure mice bearing MCA sarcomas, Renca, and other more immunogenic tumors. In addition, strategies that can render "non-immunogenic" mouse tumors more susceptible to immune destruction, such as transfection with the costimulatory molecule B7-1, tend to work much less well, if at all, in B16 (Chen et al., 1994; Wu et al., 1995). The reason for this low immunogenicity is still unknown, although the low expression of MHC Class I is one obvious candidate. Although it is possible to induce therapeutic immune responses to B16, it is useful to keep in mind that a strategy that is effective in another tumor model may not necessarily work for B16 melanoma. This tends to be one of the tougher tests for any form of immunotherapy.

How to induce immune response: Effective vaccinations

There are several incidental reports in the literature of regimens that can prevent growth of melanoma in normal C57BL/6 mice; however, in the authors' experience, many of these are poorly reproducible. For example, vaccination using irradiated B16, with or without admixture of BCG or *C. parvum*, typically does not confer protection to subsequent challenge with even a minimum tumorigenic dose (i.e., 1×10^5 cells) of B16. The transfected line, B16.B7-1, expressing the costimulatory molecule B7-1, tends to display reduced tumorigenicity but still fails to immunize against wild- type B16, probably since most of the killing of B16.B7-1 seems to be mediated through direct recognition by NK cells (Chen et al., 1994; Wu et al., 1995). However some recent approaches based on the specific targeting of the MDA, TRP-1, appear to give consistent protection and/or treatment of B16, i.e., repeated injections of monoclonal antibodies against TRP-1 (Hara et al., 1995) or vaccination with baculovirus encoding mTRP-1 (Naftzger et al., 1996), plasmid DNA encoding human TRP-1 (Weber et al., 1998), or a modified TRP-1 peptide in adjuvant (Dyall et al., 1998).

In the authors' laboratory, the following three treatment protocols reliably and reproducibly impact significantly on B16 growth.

1. Treatment of established pulmonary metastases by adoptive transfer of CTL specific for the MDA gp100 or TRP-2, or the

Table 20.1.3 B16 as a Model for Human Melanoma: Similarities and Differences

Similarities	Differences
Human melanomas express at least 5 different MDA: gp100/pmel 17, MART-1/Melan-A, tyrosinase, TRP-1/gp75, and TRP-2. All of these can be recognized by CTL from human melanoma patients: the mouse homologs of these genes are all expressed in B16 melanoma.	Human melanomas express variable levels of MHC class I; B16 murine melanoma normally expresses low levels of MHC Class I.
MDA, mgp100, mTyr, mTRP-1, and mTRP-2 have been reported to be recognized by mouse CTL (Bloom et al., 1997; Dyall et al., 1998; Overwijk et al., 1998; Colella et al., 2000)	Growing human melanoma biopsy fragments in IL-2 will yield melanoma-specific CTL in ~50% of cases; B16 biopsy fragments grown in IL-2 rarely yield B16-specific CTL.
Adoptive transfer of MDA-specific CTL can cause autoimmunity both in mice and human (Overwijk et al., 2003; Johnson et al., 2009)	Human melanomas have expressed oncogene mutations such as BRAF V600E, which can be targeted by inhibitors. B16 tumor lacks many of the mutations found in human melanomas.
Both human melanomas and B16 can often be induced to express MHC Class II by IFN-γ treatment. (Bohm et al., 1998; Surman et al., 2000)	Humans can survive for months or years despite melanoma growth; a minimal tumorigenic dose of B16 melanoma (intravenous or subcutaneous) will kill an untreated mouse within weeks.

retroviral antigen p15E (Bloom et al., 1997; Overwijk et al., 1998; Zeh III et al., 1999).

2. Protection against subcutaneous B16 challenge of mice with autoimmune depigmentation (i.e., vitiligo) after two vaccinations with recombinant vaccinia virus (rVV) encoding mouse TRP-1 (rVVmTRP-1; Overwijk et al., 1999a).

3. Protection against and partial treatment of subcutaneous B16 in mice vaccinated with irradiated B16 expressing GM-CSF (B16.GM-CSF), particularly when administered together with anti-CTLA-4 antibody (Hung et al., 1998; van Elsas et al., 1999a).

How to define the type of response induced

There are several approaches to identify the immune compartments responsible for an immune response to B16. First, purified freshly isolated or cultured lymphocyte subsets can be adoptively transferred into naive or tumor-bearing animals; examples are the transfer of purified $CD4^+$ or $CD8^+$ fresh splenocytes or the adoptive transfer of cultured, B16-specific CTL. Second, lymphocyte subpopulations can be isolated and studied for specificity toward target antigens, for example, by culturing $CD4^+$ and $CD8^+$ T lymphocytes with specific antigen and subsequently testing for antigen recognition (see Support Protocols 3 and 4). Likewise, serum from treated mice can be tested for the presence of antigen-specific antibodies (see Support Protocol 10). Third, mice can be depleted from T cell subsets using in vivo administration of antibodies specific for $CD4^+$ or $CD8^+$. Fourth, mice can be used that are deficient in certain lymphocyte subsets or immune-modulating molecules due to genetic mutations, either spontaneous, such as nude (T cells) or beige (NK cells) mice, or induced through knockout technology (such as CD4, MHC Class II, β2m, TAP-1, RAG-1, or IgM KO mice) available from commercial sources or research laboratories. Fifth, using genetic engineering to overexpress or delete genes of interest in T cells is a quick and feasible way to access their functions both in vitro and in vivo (see Basic Protocol 6 and Support Protocol 8).

Critical Parameters

One of the most important aspects of in vivo mouse experiments is ensuring that tumor take among mice is highly consistent. Inconsistent injection techniques make it very difficult to distinguish therapeutic effects over random fluctuations in tumor take. Practicing the injection of tumor, virus, and CTL is critical, not only to ensure reproducible inoculations but also to allow quick completion of the injection procedure, thus reducing the

Table 20.1.4 Troubleshooting Guide for Use of Mouse Model for Human Melanoma

Problem	Possible cause	Solution
Poor tumor take	Mycoplasma infection of tumor cultures	Eliminate mycoplasma with ciprofloxacin or thaw vial with fresh tumor sample
	Over-trypsinization of tumor cells	Dilute out trypsin with large volume of HBSS as soon as tumor cells dislodge; pellet and discard supernatant
	Tumor cells not kept on ice or on ice for a long time	Keep tumor on ice at all time and plan the experiment to minimize cell standing-by time
	Injected too few tumor cells	Increase inoculum of tumor cells
Poor treatment by adoptive transfer of CTL	Low potency or specificity of CTL	Test CTL in vitro directly prior to transfer to ensure specificity
	Low viability or too few CTL transferred	Transfer more CTL; Ficoll-enrich live cells; minimize cell stand-by time
Poor CTL growth	Missing, incorrect does or poor quality components in CM. For examples: 2-mercaptoethanol, IL-2 and FBS.	Make fresh CM/IL-2; choose an FBS lot optimal for growing mouse T cells
	Poor quality of peptide stock	Make fresh peptide in DMSO
	Over- or under-stimulation	Restimulate every 7-10 days
	Overcrowding of culture wells	Split once T cells exceed 50% confluency
Poor CTL specificity/high background (often irreversible)	Too much IL-2 in cultures	Keep IL-2 concentration between 30 and 60 IU/ml
	"Dirty culture"	Perform Ficoll separation to eliminate debris
	Progressive deterioration of specificity, large, granular, fast-growing cells	Discard culture and thaw new vial or establish new culture from immunized mice
No induction of vitiligo and/or antibodies upon two immunizations with rVVmTRP-1	Poor viral titer	Re-titer viral stock
	Mixup of rVV stocks	Check expression by immunostaining of infected cells
Large variables in tumor treatment within a group	Variable tumor size prior to treatment	Inject extra mice and select mice with similar size of tumor for the experiment

continued

Table 20.1.4 Troubleshooting Guide for Use of Mouse Model for Human Melanoma, *continued*

Problem	Possible cause	Solution
	Poorly mixed cells	Make sure the cells are mixed well before injection and do not leave cells in the syringe for a long time.
Difficult to clone full-length TCR α and β chain	PCR conditions hard to optimize	Synthesize full length cDNA using V region and constant region (one α, two βs)
Low transduction efficiency	Sub-optimized retroviral vector construct	Codon optimization of cDNAs, switch α and β chain positions.
	Poorly activated T cells	Check culture conditions and media
	Low viral titers	Perform second transduction 24 hr after the 1st one.
Poor T cell viability after retroviral transduction	High viral titers	Wash T cells with CM and split cells

time reagents remain on ice. Presence of mycoplasma in B16 cultures can also result in inconsistent tumor take. B16 is known as a relatively "unstable" tumor. B16 cultures from different laboratories can differ significantly in minimal tumorigenic dose, degree of pigmentation, expression of antigens, and growth rate in mice. Some optimization may be required; the numbers in this unit are merely guidelines. B16 is also known as a difficult tumor to treat; therefore failure of an immunotherapeutic regimen to impact on B16 growth does not necessarily imply it is immunologically ineffective. In vivo experiments should always be accompanied by some in vitro assays to evaluate the immunological effects of the experimental strategy. Further optimization may then result in therapeutic efficacy.

The counting of lung metastases, measurement of subcutaneous tumors, and scoring of vitiligo are all highly susceptible to observer bias and must be performed in a blinded fashion.

Troubleshooting

For detailed instruction on how to troubleshoot, please look to Table 20.1.4 for guidelines.

Time Considerations

In vivo experiments require careful planning since they may take several hours of uninterrupted work in order to minimize the time tumor cells or CTL remain on ice. A typical tumor-treatment experiment lasts a couple of weeks from the day of tumor inoculation. Sufficient amount of tumor cells and CTL should be ready for use on the appropriate days, especially when using cultured T cells or retrovirally transduced T cells. Many mice with complete tumor regression can be tumor-free for more than a year and have normal lifespan. However, some tumor can grow back from the original site after temporal regression. Effective treatment usually lasts a couple of months to a year. To induce vitiligo, two injections of rVVmTRP-1 are spaced at least 2 weeks apart, after which it takes another 2 to 3 weeks until mice develop detectable vitiligo and are protected from B16 tumor take. Waiting longer after onset of vitiligo before challenging causes the immune response to wane, and protection against B16 growth will be less complete.

Acknowledgements

This work was funded by the Intramural Research Program of the National Cancer Institute; we also thank the Milstein Family Foundation for their generous support.

Literature Cited

Aguirre, A.J., Bardeesy, N., Sinha, M., Lopez, L., Tuveson, D.A., Horner, J., Redston, M.S., and DePinho, R.A. 2003. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev.* 24:3112-3126.

Baral, T.N., MacKenzie, R., and Arbabi Ghahroudi, M. 2013. Single-domain antibodies and their utility. *Curr. Protoc. Immunol.* 103:2.17.1–2.17.57.

Bloom, M.B., Perry-Lalley, D., Robbins, P.F., Li, Y., El Gamil, M., Rosenberg, S.A., and Yang, J.C. 1997. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med.* 3:453-459.

Bohm, W., Thoma, S., Leithauser, F., Moller, P., Schirmbeck, R., and Reimann, J. 1998. T cell-mediated, IFN-gamma-facilitated rejection of murine B16 melanomas. *J. Immunol.* 2:897-908.

Brahmer, J.R., Tykodi, S.S., Chow, L.Q., Hwu, W.J., Topalian, S.L., Hwu, P., Drake, C.G., Camacho, L.H., Kauh, J., Odunsi, K., Pitot, H.C., Hamid, O., Bhatia, S., Martins, R., Eaton, K., Chen, S., Salay, T.M., Alaparthy, S., Grosso, J.F., Korman, A.J., Parker, S.M., Agrawal, S., Goldberg, S.M., Pardoll, D.M., Gupta, A., and Wigginton, J.M. 2012. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 26:2455-2465.

Bronte, V., Apolloni, E., Ronca, R., Zamboni, P., Overwijk, W.W., Surman, D.R., Restifo, N.P., and Zanovello, P. 2000. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res.* 2:253-258.

Chen, L., McGowan, P., Ashe, S., Johnston, J., Li, Y., Hellstrom, I., and Hellstrom, K.E. 1994. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J. Exp. Med.* 2:523-532.

Chin, L., Pomerantz, J., Polsky, D., Jacobson, M., Cohen, C., Cordon-Cardo, C., Horner, J.W., and DePinho, R.A. 1997. Cooperative effects of INK4a and ras in melanoma susceptibility in vivo. *Genes Dev.* 21:2822-2834.

Chinnasamy, D., Yu, Z., Theoret, M.R., Zhao, Y., Shrimali, R.K., Morgan, R.A., Feldman, S.A., Restifo, N.P., and Rosenberg, S.A. 2010. Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. *J. Clin. Invest.* 11:3953-3968.

Chinnasamy, N., Wargo, J.A., Yu, Z., Rao, M., Frankel, T.L., Riley, J.P., Hong, J.J., Parkhurst, M.R., Feldman, S.A., Schrump, D.S., Restifo, N.P., Robbins, P.F., Rosenberg, S.A., and Morgan, R.A. 2011. A TCR targeting the HLA-A*0201-restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. *J. Immunol.* 2:685-696.

Colella, T.A., Bullock, T.N., Russell, L.B., Mullins, D.W., Overwijk, W.W., Luckey, C.J., Pierce, R.A., Restifo, N.P., and Engelhard, V.H. 2000. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: Implications for tumor immunotherapy. *J. Exp. Med.* 7:1221-1232.

Conlon, K.C., Lugli, E., Welles, H.C., Rosenberg, S.A., Fojo, A.T., Morris, J.C., Fleisher, T.A., Dubois, S.P., Perera, L.P., Stewart, D.M., Goldman, C.K., Bryant, B.R., Decker, J.M., Chen, J., Worthy, T.A., Figg, W.D. Sr., Peer, C.J., Sneller, M.C., Lane, H.C., Yovandich, J.L., Creekmore, S.P., Roederer, M., and Waldmann, T.A. 2014. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J. Clin. Oncol.* pii:JCO.2014.57.3329 [Epub ahead of print].

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A. Jr., Butel, J.S., and Bradley, A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 6366:215-221.

Donovan, J. and Brown, P. 2006a. Handling and restraint. *Curr. Protoc. Immunol.* 73:1.3.1-1.3.6.

Donovan, J. and Brown, P. 2006b. Animal identification. *Curr. Protoc. Immunol.* 73:1.5.1-1.5.4.

Donovan, J. and Brown, P. 2006c. Parenteral injections. *Curr. Protoc. Immunol.* 73:1.6.1-1.6.10.

Donovan, J. and Brown, P. 2006d. *Euthanasia. Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* 8:3539-3543.

Dudda, J.C., Salaun, B., Ji, Y., Palmer, D.C., Monnot, G.C., Merck, E., Boudousquie, C., Utzschneider, D.T., Escobar, T.M., Perret, R., Muljo, S.A., Hebeisen, M., Rufer, N., Zehn, D., Donda, A., Restifo, N.P., Held, W., Gattinoni, L., and Romero, P. 2013. MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer. *Immunity* 4:742-753.

Dudley, M.E. and Rosenberg, S.A. 2003. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat. Rev. Cancer* 9:666-675.

Dudley, M.E., Wunderlich, J.R., Robbins, P.F., Yang, J.C., Hwu, P., Schwartzentruber, D.J., Topalian, S.L., Sherry, R., Restifo, N.P., Hubicki, A.M., Robinson, M.R., Raffeld, M., Duray, P., Seipp, C.A., Rogers-Freezer, L., Morton, K.E., Mavroukakis, S.A., White, D.E., and Rosenberg, S.A. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 5594:850-854.

Dudley, M.E., Yang, J.C., Sherry, R., Hughes, M.S., Royal, R., Kammula, U., Robbins, P.F., Huang, J., Citrin, D.E., Leitman, S.F., Wunderlich, J., Restifo, N.P., Thomasian, A., Downey, S.G., Smith, F.O., Klapper, J., Morton, K., Laurencot, C., White, D.E., and Rosenberg, S.A. 2008. Adoptive cell therapy for patients with metastatic melanoma: Evaluation of intensive myeloablative chemoradiation preparative regimens. *J. Clin. Oncol.* 32:5233-5239.

Dudley, M.E., Gross, C.A., Langhan, M.M., Garcia, M.R., Sherry, R.M., Yang, J.C., Phan, G.Q., Kammula, U.S., Hughes, M.S., Citrin, D.E., Restifo, N.P., Wunderlich, J.R., Prieto, P.A.,

Hong, J.J., Langan, R.C., Zlott, D.A., Morton, K.E., White, D.E., Laurencot, C.M., and Rosenberg, S.A. 2010. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin. Cancer Res.* 24:6122-6131.

Dyall, R., Bowne, W.B., Weber, L.W., LeMaoult, J., Szabo, P., Moroi, Y., Piskun, G., Lewis, J.J., Houghton, A.N., and Nikolic-Zugic, J. 1998. Heteroclitic immunization induces tumor immunity. *J. Exp. Med.* 9:1553-1561.

Earl, P.L., Cooper, N., Wyatt, L., Moss, B., and Carroll, M.W. 1998. *Curr. Protoc. Mol. Biol.* 60:16.16.1-16.16.19.

Fidler, I.J. and Bucana, C. 1977. Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. *Cancer Res.* 11:3945-3956.

Fidler, I.J., Gersten, D.M., and Budmen, M.B. 1976. Characterization in vivo and in vitro of tumor cells selected for resistance to syngeneic lymphocyte-mediated cytotoxicity. *Cancer Res.* 9:3160-3165.

Gattinoni, L., Finkelstein, S.E., Klebanoff, C.A., Antony, P.A., Palmer, D.C., Spiess, P.J., Hwang, L.N., Yu, Z., Wrzesinski, C., Heimann, D.M., Surh, C.D., Rosenberg, S.A., and Restifo, N.P. 2005. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J. Exp. Med.* 7:907-912.

Greenberg, N.M., DeMayo, F., Finegold, M.J., Medina, D., Tilley, W.D., Aspinall, J.O., Cunha, G.R., Donjacour, A.A., Matusik, R.J., and Rosen, J.M. 1995. Prostate cancer in a transgenic mouse. *Proc. Natl. Acad. Sci. U.S.A.* 8:3439-3443.

Hanahan, D. 1985. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 6015:115-122.

Hara, I., Takechi, Y., and Houghton, A.N. 1995. Implicating a role for immune recognition of self in tumor rejection: Passive immunization against the brown locus protein. *J. Exp. Med.* 5:1609-1614.

Hart, I.R. 1979. The selection and characterization of an invasive variant of the B16 melanoma. *Am. J. Pathol.* 3:587-600.

Hingorani, S.R., Wang, L., Multani, A.S., Combs, C., Deramaudt, T.B., Hruban, R.H., Rustgi, A.K., Chang, S., and Tuveson, D.A. 2005. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* 5:469-483.

Hinrichs, C.S., Borman, Z.A., Cassard, L., Gattinoni, L., Spolski, R., Yu, Z., Sanchez-Perez, L., Muranski, P., Kern, S.J., Logun, C., Palmer, D.C., Ji, Y., Reger, R.N., Leonard, W.J., Danner, R.L., Rosenberg, S.A., and Restifo, N.P. 2009. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* 41:17469-17474.

Hughes, M.S., Yu, Y.Y., Dudley, M.E., Zheng, Z., Robbins, P.F., Li, Y., Wunderlich, J., Hawley, R.G., Moayeri, M., Rosenberg, S.A., and Morgan, R.A. 2005. Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. *Hum. Gene Ther.* 4:457-472.

Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D., and Levitsky, H. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* 12:2357-2368.

Irvine, K.R., Chamberlain, R.S., Shulman, E.P., Surman, D.R., Rosenberg, S.A., and Restifo, N.P. 1997. Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors. *J. Natl. Cancer Inst.* 21:1595-1601.

Irvine, K.R., Parkhurst, M.R., Shulman, E.P., Tupesis, J.P., Custer, M., Touloukian, C.E., Robbins, P.F., Yafal, A.G., Greenhalgh, P., Sutmuller, R.P., Offringa, R., Rosenberg, S.A., and Restifo, N.P. 1999. Recombinant virus vaccination against "self" antigens using anchor-fixed immunogens. *Cancer Res.* 11:2536-2540.

Johnson, L.A., Morgan, R.A., Dudley, M.E., Cassard, L., Yang, J.C., Hughes, M.S., Kammula, U.S., Royal, R.E., Sherry, R.M., Wunderlich, J.R., Lee, C.C., Restifo, N.P., Schwarz, S.L., Cogdill, A.P., Bishop, R.J., Kim, H., Brewer, C.C., Rudy, S.F., VanWaes, C., Davis, J.L., Mathur, A., Ripley, R.T., Nathan, D.A., Laurencot, C.M., and Rosenberg, S.A. 2009. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 3:535-546.

Kerkar, S.P., Muranski, P., Kaiser, A., Boni, A., Sanchez-Perez, L., Yu, Z., Palmer, D.C., Reger, R.N., Borman, Z.A., Zhang, L., Morgan, R.A., Gattinoni, L., Rosenberg, S.A., Trinchieri, G., and Restifo, N.P. 2010. Tumor-specific CD8+ T cells expressing interleukin-12 eradicate established cancers in lymphodepleted hosts. *Cancer Res.* 17:6725-6734.

Klebanoff, C.A., Finkelstein, S.E., Surman, D.R., Lichtman, M.K., Gattinoni, L., Theoret, M.R., Grewal, N., Spiess, P.J., Antony, P.A., Palmer, D.C., Tagaya, Y., Rosenberg, S.A., Waldmann, T.A., and Restifo, N.P. 2004. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proc. Natl. Acad. Sci. U.S.A.* 7:1969-1974.

Kochenderfer, J.N., Yu, Z., Frasheri, D., Restifo, N.P., and Rosenberg, S.A. 2010. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood* 19:3875-3886.

Kochenderfer, J.N., Dudley, M.E., Carpenter, R.O., Kassim, S.H., Rose, J.J., Telford, W.G., Hakim, F.T., Halverson, D.C., Fowler, D.H., Hardy, N.M., Mato, A.R., Hickstein, D.D., Gea-Banacloche, J.C., Pavletic, S.Z., Sportes, C., Maric, I., Feldman, S.A., Hansen, B.G., Wilder, J.S., Blacklock-Schuver, B., Jena, B., Bishop, M.R., Gress, R.E., and Rosenberg, S.A. 2013.

Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood* 25:4129-4139.

Kruisbeek, A. M. 2000. Isolation of mouse mononuclear cells. *Curr. Protoc. Immunol.* 39:3.1.1-3.1.5.

Li, M., Xu, F., Muller, J., Hearing, V.J., and Gorelik, E. 1998. Ecotropic C-type retrovirus of B16 melanoma and malignant transformation of normal melanocytes. *Int. J. Cancer* 3:430-436.

Lindsey, K.R., Gritz, L., Sherry, R., Abati, A., Fetsch, P.A., Goldfeder, L.C., Gonzales, M.I., Zinnack, K.A., Rogers-Freezer, L., Haworth, L., Mavroukakis, S.A., White, D.E., Steinberg, S.M., Restifo, N.P., Panicali, D.L., Rosenberg, S.A., and Topalian, S.L. 2006. Evaluation of prime/boost regimens using recombinant poxvirus/tyrosinase vaccines for the treatment of patients with metastatic melanoma. *Clin. Cancer Res.* 8:2526-2537.

Morgan, R.A., Dudley, M.E., Wunderlich, J.R., Hughes, M.S., Yang, J.C., Sherry, R.M., Royal, R.E., Topalian, S.L., Kammula, U.S., Restifo, N.P., Zheng, Z., Nahvi, A., de Vries, C.R., Rogers-Freezer, L.J., Mavroukakis, S.A., and Rosenberg, S.A. 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 5796:126-129.

Morgan, R.A., Chinnasamy, N., Abate-Daga, D., Gros, A., Robbins, P.F., Zheng, Z., Dudley, M.E., Feldman, S.A., Yang, J.C., Sherry, R.M., Phan, G.Q., Hughes, M.S., Kammula, U.S., Miller, A.D., Hessman, C.J., Stewart, A.A., Restifo, N.P., Quezado, M.M., Alimchandani, M., Rosenberg, A.Z., Nath, A., Wang, T., Bielekova, B., Wuest, S.C., Akula, N., McMahon, F.J., Wilde, S., Mosetter, B., Schendel, D.J., Laurencot, C.M., and Rosenberg, S.A. 2013. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J. Immunother.* 2:133-151.

Muranski, P., Boni, A., Antony, P.A., Cassard, L., Irvine, K.R., Kaiser, A., Paulos, C.M., Palmer, D.C., Touloukian, C.E., Ptak, K., Gattinoni, L., Wrzesinski, C., Hinrichs, C.S., Kerstann, K.W., Feigenbaum, L., Chan, C.C., and Restifo, N.P. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 2:362-373.

Naftzger, C., Takechi, Y., Kohda, H., Hara, I., Vijayasaradhi, S., and Houghton, A.N. 1996. Immune response to a differentiation antigen induced by altered antigen: A study of tumor rejection and autoimmunity. *Proc. Natl. Acad. Sci. U.S.A.* 25:14809-14814.

Otsuka, T., Takayama, H., Sharp, R., Celli, G., LaRochelle, W.J., Bottaro, D.P., Ellmore, N., Vieira, W., Owens, J.W., Anver, M., and Merlino, G. 1998. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. *Cancer Res.* 22:5157-5167.

Overwijk, W.W., Surman, D.R., Tsung, K., and Restifo, N.P. 1997. Identification of a Kb-restricted CTL epitope of beta-galactosidase: potential use in development of immunization protocols for "self" antigens. *Methods* 2:117-123.

Overwijk, W.W., Tsung, A., Irvine, K.R., Parkhurst, M.R., Goletz, T.J., Tsung, K., Carroll, M.W., Liu, C., Moss, B., Rosenberg, S.A., and Restifo, N.P. 1998. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med.* 2:277-286.

Overwijk, W.W., Lee, D.S., Surman, D.R., Irvine, K.R., Touloukian, C.E., Chan, C.C., Carroll, M.W., Moss, B., Rosenberg, S.A., and Restifo, N.P. 1999a. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 6:2982-2987.

Overwijk, W.W., Lee, D.S., Surman, D.R., Irvine, K.R., Touloukian, C.E., Chan, C.C., Carroll, M.W., Moss, B., Rosenberg, S.A., and Restifo, N.P. 1999b. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: Requirement for CD4(+) T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 6:2982-2987.

Overwijk, W.W., Theoret, M.R., Finkelstein, S.E., Surman, D.R., de Jong, L.A., Vyth-Dreese, F.A., Dellemijn, T.A., Antony, P.A., Spiess, P.J., Palmer, D.C., Heimann, D.M., Klebanoff, C.A., Yu, Z., Hwang, L.N., Feigenbaum, L., Kruisbeek, A.M., Rosenberg, S.A., and Restifo, N.P. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J. Exp. Med.* 4:569-580.

Parkhurst, M.R., Joo, J., Riley, J.P., Yu, Z., Li, Y., Robbins, P.F., and Rosenberg, S.A. 2009. Characterization of genetically modified T-cell receptors that recognize the CEA:691-699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. *Clin. Cancer Res.* 1:169-180.

Parkhurst, M.R., Yang, J.C., Langan, R.C., Dudley, M.E., Nathan, D.A., Feldman, S.A., Davis, J.L., Morgan, R.A., Merino, M.J., Sherry, R.M., Hughes, M.S., Kammula, U.S., Phan, G.Q., Lim, R.M., Wank, S.A., Restifo, N.P., Robbins, P.F., Laurencot, C.M., and Rosenberg, S.A. 2011. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol. Ther.* 3:620-626.

Phan, G.Q. and Rosenberg, S.A. 2013. Adoptive cell transfer for patients with metastatic melanoma: The potential and promise of cancer immunotherapy. *Cancer Control* 4:289-297.

Phan, G.Q., Yang, J.C., Sherry, R.M., Hwu, P., Topalian, S.L., Schwartzentruber, D.J., Restifo, N.P., Haworth, L.R., Seipp, C.A., Freezer, L.J., Morton, K.E., Mavroukakis, S.A., Duray, P.H., Steinberg, S.M., Allison, J.P., Davis, T.A., and Rosenberg, S.A. 2003. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. U.S.A.* 14:8372-8377.

Rabinovich, B.A., Ye, Y., Etto, T., Chen, J.Q., Levitsky, H.I., Overwijk, W.W., Cooper, L.J., Gelovani, J., and Hwu, P. 2008. Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferase in immunocompetent mouse models of cancer. *Proc. Natl. Acad. Sci. U.S.A.* 38:14342-14346.

Robbins, P.F., Morgan, R.A., Feldman, S.A., Yang, J.C., Sherry, R.M., Dudley, M.E., Wunderlich, J.R., Nahvi, A.V., Helman, L.J., Mackall, C.L., Kammula, U.S., Hughes, M.S., Restifo, N.P., Raffeld, M., Lee, C.C., Levy, C.L., Li, Y.F., El Gamil, M., Schwarz, S.L., Laurencot, C., and Rosenberg, S.A. 2011. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J. Clin. Oncol.* 7:917-924.

Rosenberg, S.A. 1999. A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity* 3:281-287.

Rosenberg, S.A., Spiess, P., and Lafreniere, R. 1986. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 4770:1318-1321.

Rosenberg, S.A., Yang, J.C., Schwartzentruber, D.J., Hwu, P., Marincola, F.M., Topalian, S.L., Restifo, N.P., Dudley, M.E., Schwarz, S.L., Spiess, P.J., Wunderlich, J.R., Parkhurst, M.R., Kawakami, Y., Seipp, C.A., Einhorn, J.H., and White, D.E. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* 3:321-327.

Rosenberg, S.A., Yang, J.C., Schwartzentruber, D.J., Hwu, P., Topalian, S.L., Sherry, R.M., Restifo, N.P., Wunderlich, J.R., Seipp, C.A., Rogers-Freezer, L., Morton, K.E., Mavroukakis, S.A., Gritz, L., Panicali, D.L., and White, D.E. 2003. Recombinant fowlpox viruses encoding the anchor-modified gp100 melanoma antigen can generate antitumor immune responses in patients with metastatic melanoma. *Clin. Cancer Res.* 8:2973-2980.

Rosenberg, S.A., Restifo, N.P., Yang, J.C., Morgan, R.A., and Dudley, M.E. 2008. Adoptive cell transfer: A clinical path to effective cancer immunotherapy. *Nat. Rev. Cancer* 4:299-308.

Schwartzentruber, D.J., Lawson, D.H., Richards, J.M., Conry, R.M., Miller, D.M., Treisman, J., Gailani, F., Riley, L., Conlon, K., Pockaj, B., Kendra, K.L., White, R.L., Gonzalez, R., Kuzel, T.M., Curti, B., Leming, P.D., Whitman, E.D., Balkissoon, J., Reintgen, D.S., Kaufman, H., Marincola, F.M., Merino, M.J., Rosenberg, S.A., Choyke, P., Vena, D., and Hwu, P. 2011. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N. Engl. J. Med.* 22:2119-2127.

Small, E.J., Tchekmedyian, N.S., Rini, B.I., Fong, L., Lowy, I., and Allison, J.P. 2007. A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* 6:1810-1815.

Stewart, T.A., Pattengale, P.K., and Leder, P. 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell* 3:627-637.

Strober, W. 1997a. Monitoring cell growth. *Curr. Protoc. Immunol.* 21:3A.1-A.3A.2.

Strober, W. 1997b. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* 21:A.3B.1–A.3B.2.

Surman, D.R., Dudley, M.E., Overwijk, W.W., and Restifo, N.P. 2000. Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J. Immunol.* 2:562-565.

Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., Atkins, M.B., Leming, P.D., Spigel, D.R., Antonia, S.J., Horn, L., Drake, C.G., Pardoll, D.M., Chen, L., Sharfman, W.H., Anders, R.A., Taube, J.M., McMiller, T.L., Xu, H., Korman, A.J., Jure-Kunkel, M., Agrawal, S., McDonald, D., Kollia, G.D., Gupta, A., Wigginton, J.M., and Sznol, M. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 26:2443-2454.

van Elsas, A., Hurwitz, A.A., and Allison, J.P. 1999a. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J. Exp. Med.* 3:355-366.

van Elsas, A., Hurwitz, A.A., and Allison, J.P. 1999b. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J. Exp. Med.* 3:355-366.

van Elsas, A., Sutmuller, R.P., Hurwitz, A.A., Ziskin, J., Villasenor, J., Medema, J.P., Overwijk, W.W., Restifo, N.P., Melief, C.J., Offringa, R., and Allison, J.P. 2001. Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: Comparison of prophylaxis and therapy. *J. Exp. Med.* 4:481-489.

Voytas, D. 1991. Agarose gel electrophoresis. *Curr. Protoc. Immunol.* 2:10.4.1-10.4.8.

Weber, L.W., Bowne, W.B., Wolchok, J.D., Srinivasan, R., Qin, J., Moroi, Y., Clynes, R., Song, P., Lewis, J.J., and Houghton, A.N. 1998. Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J. Clin. Invest.* 6:1258-1264.

Wrzesinski, C., Paulos, C.M., Gattinoni, L., Palmer, D.C., Kaiser, A., Yu, Z., Rosenberg, S.A., and

Restifo, N.P. 2007. Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells. *J. Clin. Invest.* 2:492-501.

Wrzesinski, C., Paulos, C.M., Kaiser, A., Muranski, P., Palmer, D.C., Gattinoni, L., Yu, Z., Rosenberg, S.A., and Restifo, N.P. 2010. Increased intensity lymphodepletion enhances tumor treatment efficacy of adoptively transferred tumor-specific T cells. *J. Immunother.* 1:1-7.

Wu, T.C., Huang, A.Y., Jaffee, E.M., Levitsky, H.I., and Pardoll, D.M. 1995. A reassessment of the role of B7-1 expression in tumor rejection. *J. Exp. Med.* 5:1415-1421.

Xu, F., Carlos, T., Li, M., Sanchez-Sweatman, O., Khokha, R., and Gorelik, E. 1998. Inhibition of VLA-4 and up-regulation of TIMP-1 expression in B16BL6 melanoma cells transfected with MHC class I genes. *Clin. Exp. Metastasis* 4:358-370.

Yu, Z., Theoret, M.R., Touloukian, C.E., Surman, D.R., Garman, S.C., Feigenbaum, L., Baxter, T.K., Baker, B.M., and Restifo, N.P. 2004. Poor immunogenicity of a self/tumor antigen derives from peptide-MHC-I instability and is independent of tolerance. *J. Clin. Invest.* 4:551-559.

Zeh, H.J. III, Perry-Lalley, D., Dudley, M.E., Rosenberg, S.A., and Yang, J.C. 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol.* 2:989-994.

Zhu, H., Reuhl, K., Zhang, X., Botha, R., Ryan, K., Wei, J., and Chen, S. 1998. Development of heritable melanoma in transgenic mice. *J. Invest. Dermatol.* 3:247-252.