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B16 as a Mouse Model for Human Melanoma

Willem W. Overwijk and **Nicholas P. Restifo**
National Cancer Institute, NIH Bethesda, Maryland

Malignant melanoma is the sixth most common cancer in the U.S., with an estimated 44,200 new cases reported in 1999 (Centers for Disease Control and Prevention, 1999). A subset of patients with metastatic melanoma can be 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, 1997; Rosenberg, 1999). Recently, a number of different laboratories have used these anti-tumor lymphocytes to clone melanoma-associated antigens, which have generally been nonmutated melanocyte differentiation antigens (MDA), a group that includes MART-1/Melan-A, gp100, tyrosinase, and tyrosinase related proteins (TRP)-1 and -2 (Rosenberg, 1997; Rosenberg, 1999), and others listed in Table 20.1.1. With the possible exception of MART-1/Melan-A, each member of this group of proteins is an enzyme directly involved in the synthesis of eumelanin pigment (Winder et al., 1994; Kameyama et al., 1995).

Since MDA are expressed by most melanoma cells, they are an attractive target for melanoma vaccines. However, from an immunological perspective, MDA are “self” proteins, to which central and/or peripheral tolerance may exist, potentially hampering the induction of powerful, therapeutic anti-melanoma immune responses. Yet clinical observations suggest that some degree of autoreactivity can be induced and may even contribute to prolonged survival of patients. In a prospective study of patients receiving IL-2, vitiligo was seen in ~20% of melanoma patients that had objective responses. None of the nonresponding patients developed vitiligo, nor did any of the more than 100 patients receiving IL-2 for the treatment of kidney cancer (Rosenberg and White, 1996).

These observations prompted the authors of this unit to investigate the anti-tumor effect of inducing autoimmune responses to the mouse homologs of human MDA. Several models of melanoma exist in common mouse strains, including BALB/c and C57BL/6 (Donawho et al., 1996; Overwijk et al., 1998; Overwijk et al., 1999). As a model system, the authors used the spontaneous C57BL/6-derived B16 melanoma, a well established and widely used tumor model in which treatment is notoriously difficult. Results by the authors and others indicate that autoimmunity to MDA can indeed be induced, and can result in protection against and/or treatment of B16 melanoma (Naftzger et al., 1996; Bloom et al., 1997; Overwijk et al., 1998; Overwijk et al., 1999).

This unit will detail protocols for in vivo models of subcutaneous growth and pulmonary metastases of B16 melanoma (see Basic Protocols 1 and 2). Therapeutic approaches include the use of B16.GM-CSF (see Basic Protocol 3) and rVVmTRP-1 (see Basic Protocol 4) to induce autoimmune vitiligo and tumor protection. The induction and use of gp100-specific therapeutic cytotoxic T lymphocytes (CTL) are discussed. Methods are also included for CTL induction (see Support Protocol 3), isolation and testing (see Support Protocol 4), CTL maintenance (see Support Protocol 5), and adoptive transfer (see Basic Protocol 5). Support Protocols 7 and 8 detail the testing of mouse sera for presence of MDA-specific antibodies by immunoblotting and ELISA, respectively. Additional sections, including growing B16 melanoma (see Support Protocol 1), enumerating pulmonary metastases (see Support Protocol

2), and use of recombinant viruses for vaccination, are discussed together with safety concerns (see Support Protocol 6).

MOUSE MODEL OF SUBCUTANEOUS MELANOMA

The subcutaneous model is widely used for the evaluation of therapy in many tumor models, including B16 melanoma. Upon subcutaneous injection, B16 will form a palpable tumor in 5 to 10 days and grow to a 1 × 1 × 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 1×10^5 cells/mouse, which is 1.5 to 2 times the minimal tumorigenic dose in normal C57BL/6 mice. 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% confluent (see Support Protocol 1)

Trypsin/EDTA (Life Technologies)

Complete medium (CM; see recipe), 4°C

Hanks' balanced salt solution (HBSS; APPENDIX 2A), ice cold

6- to 12-week-old female C57BL/6 mice

70% ethanol

50-ml conical centrifuge tubes

Centrifuge and Sorvall H-2000B rotor, 4°C

Disposable cell strainer (Falcon)

1-ml disposable syringes and 27½-G needles

Calipers

Additional reagents and equipment for trypsinizing cells, counting cells in a hemacytometer, and determining viability by trypan blue exclusion (APPENDIX 3B), restraint of mice (UNIT 1.3), ear tagging (UNIT 1.5), and subcutaneous injection of mice (UNIT 1.6)

Prepare B16 cells

1. Ensure that B16 cells are in the logarithmic growth phase when harvesting for injection, i.e., flasks should be ≤50% confluent.

   Nondividing tumor cells from confluent flasks may take less well.

2. Aspirate medium, rinse flask briefly with 3 ml trypsin/EDTA, and aspirate again.

   Rinsing helps remove fetal bovine serum (FBS), which otherwise dilutes the trypsin and inhibits proteolysis. APPENDIX 3B provides additional detail on trypsinization of cells.

3. Add 5 ml trypsin/EDTA and tilt flask to ensure that all cells are covered. Periodically, 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 complete medium and pipet vigorously to obtain single-cell suspension.

5. Transfer to 50-ml conical centrifuge tube and add 40 ml cold CM to neutralize trypsin. Pellet cells for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

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

7. Pass suspension through disposable cell strainer to remove any clumps. Count live cells using trypan blue (*APPENDIX 3B*).

Viability should be >90%.

8. Adjust cell concentration to 1 × 10^6 cells/ml in ice-cold HBSS.

Inoculate mice

9. Relocate work area to facility where 6 to 12 week old female C57BL/6 mice are kept, maintaining cells on ice.

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

10. Resuspend cells by inverting tube several times. Fill 1-ml syringe with attached 27½-G needle.

11. Wet abdominal fur with 1 or 2 drops 70% ethanol, rub fur in a downward manner with one finger, then part fur in the middle with needle.

"Swipe" the hairs sideways by moving the needle perpendicular to the abdomen, parting the hairs so that the skin becomes clearly visible.

12. Insert needle very superficially, so that it is visible through the semi-transparent skin.

This requires some practice; the key is remaining very superficial without reemerging through the skin. UNIT 1.6 provides additional information on subcutaneous injections.

13. Slide needle 5 to 10 mm subcutaneously and inject 100 μl cell suspension; watch for appearance of a "bleb."

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 in cage.

Discard the last 0.5 ml in the syringe; typically tumor cells collect against the plunger, resulting in a disproportionately large amount of tumor cells in the last injection.

Randomize mice and follow up

15. Ear tag mice (*UNIT 1.5*) 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. Wetting fur with 70% ethanol facilitates early detection.

MOUSE MODEL OF MULTIPLE PULMONARY MELANOMA METASTASES

The pulmonary metastasis model is the other widely used model for the evaluation of 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 is amelanotic ("white"); this requires careful counting in order not to underestimate tumor burden.

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

Materials

B16 culture, ≤50% confluent (see Support Protocol 1)

Trypsin/EDTA (Life Technologies)

Complete medium (CM; see recipe), 4°C

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)

6- to 12-week-old female C57BL/6 mice

50-ml conical centrifuge tubes

Centrifuge and Sorvall H-2000B rotor, 4°C

Disposable cell strainer (Falcon)

Heat lamp

3-ml disposable syringes and 27 1/2-G needles

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3B*), restraint of mice (*UNIT 1.3*), ear tagging (*UNIT 1.5*), and intravenous injections (*UNIT 1.6*)

Prepare B16 cells

1. Perform steps 1 to 7 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 HBSS.

Inoculate mice

3. Relocate work area to facility where 6 to 12 week old female C57BL/6 mice are kept, maintaining 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 3-ml syringe with B16 cells and attach a 27½-G needle.

   Failure to resuspend will result in steadily decreasing numbers of tumor cells injected.

6. Restrain mice using a restrainer that allows the tail to protrude and inject 0.5 ml cells intravenously in tail vein (also see UNIT 1.6).

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

7. Continue to resuspend cells before each syringe refill.

Discard the last 0.5 ml in the syringe; typically tumor cells collect against the plunger, resulting in a disproportionately large amount of tumor cells in the last injection.

Randomize mice and follow-up

8. If mice have not been pretreated, randomize mice after they have all been injected with B16 cells and ear tag mice (UNIT 1.5) to blind the experiment.

9. Euthanize mice by CO_2 asphyxiation 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.

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

It is difficult to induce reliable protection against B16 challenge by vaccination with irradiated B16, even when admixed with *Corynebacterium parvum.* However, robust protection can be obtained by vaccinating with B16 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. It is unknown what antigens are targets of this 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. Recent results suggest it may also be possible to impact on growth of established tumors by vaccinations with irradiated B16.GM-CSF, especially in conjunction with anti-CTLA-4 antibody (van Elsas et al., 1999). 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% confluent (see Support Protocol 1)

B16 culture, ≤50% confluent (see Support Protocol 1)

Trypsin/EDTA (Life Technologies)

Complete medium (CM; see recipe), 4°C

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), ice cold

6- to 12-week-old female C57BL/6 mice

50-ml conical centrifuge tubes

Centrifuge and Sorvall H-2000B rotor, 4°C

Disposable cell strainer (Falcon)

3-ml disposable syringes and 27½-G needles

γ irradiator

Calipers

Additional reagents and equipment for trypsinizing cells, counting cells in a hemacytometer, and determining viability by trypan blue exclusion (*APPENDIX 3B*), restraint of mice (*UNIT 1.3*), ear tagging (*UNIT 1.5*), and intravenous injections (*UNIT 1.6*)

Prepare B16.GM-CSF vaccine

1. Ensure B16.GM-CSF cells are in the logarithmic growth phase when harvesting for injection, i.e., flasks should be ≤50% confluent.

2. Aspirate medium and rinse flask briefly with 3 ml trypsin/EDTA and aspirate.

Rinsing helps remove fetal bovine serum (FBS) which otherwise dilutes the trypsin and competes with cell adhesion molecules for proteolysis. APPENDIX 3B provides additional detail on trypsinization of cells.

3. Add 5 ml trypsin/EDTA, then tilt flask to ensure that all cells are covered. Periodically, 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 CM and pipet vigorously to obtain single-cell suspension.

5. Transfer to 50-ml conical centrifuge tube and add 40 ml cold CM to neutralize trypsin. Centrifuge cells for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

6. Decant supernatant and resuspend cells in ice-cold HBSS, aiming at $>1\times10^7$ cells/ml.

7. Pass suspension through disposable cell strainer to remove any clumps. Count live cells using trypan blue (*APPENDIX 3B*).

Viability should be >90%.

8. Adjust cell concentration to 1×10^7/ml in ice-cold HBSS.

9. Irradiate cells (i.e., 5000 rad).

Vaccinate mice

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

11. Inject 100 μl (1×10^6 cells) of the irradiated B16.GM-CSF subcutaneously as described in Basic Protocol 1, steps 9 to 14.

Challenge mice

12. Two to 3 weeks after vaccination, challenge mice with 1×10^5 wild-type (untransduced) B16 as described in Basic Protocol 1.

Randomize mice and follow-up

13. Ear tag mice (*UNIT 1.5*) to blind the experiment and randomize over cages.

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

Tumors in control mice should become palpable in 5 to 10 days; wetting fur with 70% ethanol facilitates early detection.

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

Currently, the two tumor vaccines that induce the most reliable protection of mice from lethal B16 challenge are rVV encoding the MDA, mTRP-1 and irradiated B16 expressing GM-CSF (Hung et al., 1998; Overwijk et al., 1999). Although immunization with rVVhgp100 induces high levels of gp100-specific 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 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 adenoviruses cause 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 administered 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 vitiglio 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 or 70% alcohol first 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

Additional reagents and equipment for immunization of mice with recombinant viral vectors (see Support Protocol 6)

Immunize mice

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

   Prepare rVV for intravenous injection according to instructions in Support Protocol 6.

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 apparent depigmentation.

   For photographs of typical results, see Figure 1 in Overwijk et al. (1999).

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 B16 melanoma and follow tumor growth (see Basic Protocol 1).

Monitor serological response

5. Collect serum from immunized mice at any time for testing by immunoblot analysis (see Support Protocol 7) or ELISA (see Support Protocol 8).

   Serum can be collected repeatedly from the retro-orbital space or by nicking (not amputating) the ventral base of the tail.

ADOPTIVE TRANSFER OF B16 MELANOMA-SPECIFIC CTL TO TREAT ESTABLISHED PULMONARY METASTASES

Adoptive transfer of MDA-specific CTL works well in the B16 pulmonary metastases model (Overwijk et al., 1998; Bloom et al., 1997). Adoptive transfer of CTL in mice bearing subcutaneous B16 has little to no effect. This disparity is also seen in other mouse tumor models such as MCA-induced sarcomas, and seems to be a general feature when using cultured $CD8^+$ T cells. One possible reason may be the apparent preferential location of both tumor cells and CTL to the lungs upon intravenous injection. It is important to keep in mind that successful adoptive therapy is typically much easier to achieve in mice with pulmonary disease than in mice with subcutaneous tumors.

CTL are best used for adoptive transfer 5 days or more after the last restimulation, to prevent nonspecific activity. To ensure the quality of CTL, they can be tested in a small in vitro assay using B16 and several antigen-negative tumor cells as controls. If available, specific peptides should be included as targets (see Support Protocol 4). As an additional control, CTL with a specificity other than B16 can be transferred into B16-bearing mice to ensure that any tumor killing is not a result of antigen nonspecific cytotoxicity or cytokine release.

Cell number is an important factor in adoptive transfer; numbers too low may be ineffective whereas numbers too high could result in nonspecific killing, although the latter is rarely observed. Nonspecific killing is controlled for by including a nonspecific CTL line and/or mice bearing an antigen-negative tumor instead of B16. Depending on the CTL line, a typical cell number is $2–10 \times 10^6$ CTL per mouse when rIL-2 is added. The addition of rIL-2 can diminish the required number of CTL by 2 to 10 fold, but is not necessary. A typical adoptive transfer is performed 3 days after tumor inoculation. The ability to treat pulmonary disease rapidly decreases with increasing time between tumor inoculation and adoptive transfer.

Materials

B16-specific CTL (e.g., gp100-specific CTL), ≥5 days post last restimulation (see Support Protocol 3)

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), ice cold

6 to 12 week old female C57BL/6 mice

50-ml conical centrifuge tubes

Centrifuge and Sorvall H-2000B rotor, 4°C

Heat lamp

Restrainer

3-ml disposable syringes and 27 1/2-G needles

Additional reagents and equipment for counting cells in a hemacytometer and determining viability by trypan blue exclusion (*APPENDIX 3B*), restraint of mice (*UNIT 1.3*), ear tagging (*UNIT 1.5*), intravenous injections (*UNIT 1.6*), and euthanasia by CO_2 asphyxiation (*UNIT 1.8*)

Prepare CTL

1. Harvest B16-specific CTL that were last restimulated ≥5 days ago by pipetting wells up and down and transfer to 50-ml conical centrifuge tube.

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

2. Pellet CTL 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C. Resuspend CTL in ice-cold HBSS, aiming for a final concentration of 2×10^7 cells/ml.

If a lot of debris is present in cultures, purify CTL on a Ficoll gradient according to manufacturer's protocol (see Support Protocol 3).

Debris will cause lethal embolisms upon intravenous injection.

3. Count CTL using trypan blue to exclude dead cells (*APPENDIX 3B*).

4. Dilute CTL to desired concentration, keeping in mind that each mouse is to receive 0.5 ml CTL suspension. Keep on ice.

Transfer CTL

5. Using 6- to 12-week old female C57BL/6 mice bearing 3-day B16 pulmonary metastases (see Basic Protocol 2), randomize mice by pooling in large cage.

6. Warm mice by placing under heat lamp for 2 to 3 min until tail veins are dilated. Restrain mice using a restrainer that allows tail to protrude.

7. Resuspend CTL before injection by repeatedly inverting tube. Inject 0.5 ml CTL suspension intravenously in tail vein using a 3-ml syringe and 27½-G needle.

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

Optional: inject 60,000 to 600,000 IU rhIL-2 in 0.5 ml PBS intravenously two or three times per day for 5 days or until mice show signs of toxicity (ruffled fur, hunched posture).

IL-2-induced toxicity at the highest doses can be severe and lethal but is reversible in one day after discontinued administration. If IL-2 is withheld for one group it is best to withhold for all to allow proper comparison between groups. “A living mouse receiving most of its IL-2 is worth more than a dead mouse that got all its shots.”

Sacrifice and evaluate pulmonary tumor burden

8. Euthanize mice by CO_2 asphyxiation 16 to 18 days after tumor inoculation and enumerate pulmonary metastases (see Support Protocol 2). Alternatively, follow mice for survival.

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

GROWING B16 MELANOMA

Many laboratories carry B16 lines; however it is important to note that B16 tends to be an unstable cell line that knows 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, minimal 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. B16.F10 and other sublines can be obtained from

American Type Tissue Collection (ATCC; see *SUPPLIERS APPENDIX*) 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.

NOTE: All solutions are prepared using double-distilled or Milli-Q-filtered (Millipore) water and are sterile filtered through 0.2-um filter units (Nalgene). All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator.

Materials

Cryopreserved vial of B16.F10

Complete medium (CM; see recipe), ice cold

15-ml tubes

175- and 162-cm^2 tissue culture coated flasks

Humidified 37°C, 5% CO_2 incubator

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3B*)

Thaw and plate B16 cells

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

   Prevent "heat shock" to tumor cells.

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

3. Pellet cells for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

   Cell pellet should appear light brown to black.

4. Decant supernatant and resuspend cells in 20 ml ice-cold CM.

5. Plate in 175-cm^2 tissue culture coated flask and grow in 37°C, 5% CO_2 incubator.

Maintain cultures

6. Split 1:5 to 1:10 into a 162-cm^2 tissue culture flask when cells are 50% confluent (typically twice a week).

7. Use cells when in logarithmic growth phase (≤50% confluent); this will yield 1–5 × 10^7 cells per 162-cm^2 flask.

ENUMERATING PULMONARY METASTASES IN MICE WITH B16 MELANOMA

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 1)

Fekete's solution (see recipe)

Hardboard plate and pins

Sterile dissecting tools

Scintillation vials (PGC Scientifics)

Additional reagents and equipment for ear tagging (*UNIT 1.5*) and euthanasia by CO_2 asphyxiation (*UNIT 1.8*)

Isolate lungs

1. Ear tag mice (*UNIT 1.5*), if necessary.

2. Euthanize mice by CO_2 asphyxiation.

   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 to 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 scissors, taking care not to cut into lungs.

5. Using forceps, 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 water to remove excess blood.

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

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

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

Enumerate nodules

10. Enumerate pulmonary nodules blindly.

11. Using forceps, pry fixed lungs apart into individual lobes and enumerate tumor nodules on each lung blindly, 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.

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 (see Table 20.1.1 for a note on preparing peptide stocks). 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 et al., 1999). This protocol has been used successfully to determine efficacy of immunization with recombinant viruses (i.e., rVV, rFPV, rAd; see Support Protocol 6), whole tumor cells (see Basic Protocol 3), 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)

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), ice cold

Complete medium (CM; see recipe), ice cold

Stimulator peptide stocks (see Table 20.1.1)

Sterile dissecting instruments

60-mm-diameter polystyrene petri dishes

3- or 6-ml syringe

15-ml centrifuge tube

Disposable cell strainer (Falcon)

Centrifuge Sorvall H-2000B rotor, 4°C

25-cm^2 tissue culture flasks

Additional reagents and equipment for preparing spleen cell suspensions (*UNIT 3.1*) and euthanasia by CO_2 asphyxiation (*UNIT 1.8*)

Isolate splenocytes

1. Euthanize mice using CO_2 asphyxiation (*UNIT 1.8*).

   Use at least two vaccinated mice per group.

2. In laminar flow hood, using sterile instruments, isolate spleens into 15-ml centrifuge 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% ethanol.

3. Transfer spleen to petri dish with 1 ml ice-cold CM and mash spleens thoroughly with sterile butt end of 3- or 6-ml syringe.

   If red blood cell lysis is desired, transfer spleen to 1 ml ice-cold ACK lysing buffer (UNIT 3.1) for 2 min followed by addition of 9 volumes CM to stop RBC lysis.

   UNIT 3.1 contains additional detail on preparation of spleen cell suspensions.

4. Transfer splenocytes into 15-ml centrifuge tube and add CM to 10 ml final volume. Pipet vigorously to obtain single-cell suspension.

5. Pass cells through cell strainer into new 15-ml centrifuge tube. Pellet cells for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

6. Decant supernatant and resuspend pellet in 10 ml CM and count cells.

   Total cell number will be ~2×10^8 cells total (~1×10^8 per spleen).

   Pass through cell strainer if new clumps have formed.

7. Divide cells into groups—e.g., specific peptide(s), control peptide(s)—and culture ~2×10^6 cells/ml CM in small 25-cm^2 tissue culture flasks.

   Typically 10 to 30 ml CM/flask. 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

8. Add 1 to 10 μM final concentration of the appropriate stimulator peptide (Table 20.1.1).

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

9. Incubate flasks upright. Culture for 6 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

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 quite 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 6-day culture of the splenocytes with a different peptide, or no peptide at all. 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-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. See Table 20.1.1 for a note on preparing peptide stocks.

Materials

B16-specific CTL (see Support Protocol 3)

Complete medium (CM; see recipe)

Target cells, including B16 and control tumor cell lines

10 mg/ml stimulator peptide stock, in DMSO (see Table 20.1.1)

IFNγ and GM-CSF-specific ELISA kits (e.g., Endogen)

Centrifuge and Sorvall H-2000B rotor, room temperature and 4°C

Round-bottom 96-well plates

Additional reagents and equipment for counting cells in a hemacytometer and determining viability by trypan blue exclusion (*APPENDIX 3B*)

Harvest CTL cultures

1. Suspend B16-specific CTL from wells or flasks by vigorously pipetting medium up and down.

2. Harvest CTL by centrifuging for 10 min at 663 × *g* (in a Sorvall H-2000B 1500 rpm), room temperature.

Recovery of CTL is typically 20% of what was put into culture.

3. Decant supernatant and resuspend pellet in CM, final concentration 1–5 × 10^6 cells/ml.

4. Count CTL using trypan blue to exclude dead cells. Dilute CTL to 5 × 10^5 cells/ml in CM.

There will be quite a few dead cells.

Adjust the plane of focus to identify and exclude the “donut-shaped” RBC.

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

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

Prepare targets

6. Prepare target cells at 5 × 10^5 cells/ml.

Use B16 and a few antigen-negative control tumor lines.

7. For peptide-pulsed targets, incubate target cells with 1 to 10 μM peptide in CM at 37° C for 1 hr. Wash in CM for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C, to remove free peptide. Repeat 2 times, for a total of 3 washes.

Use like tumor cells targets.

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 readout than using peptide-pulsed tumor targets; it also eliminates possible background recognition of the tumor target itself.

Incubate CTL and targets

8. Incubate CTL with targets 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 readout.

Test supernatants

9. 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.

10. Perform IFNγ and/or GM-CSF ELISA on supernatants according to manufacturer's recommendations provided with kit.

As a readout, IFN-γ is typically more sensitive. GM-CSF can also be used but some mouse tumors constitutively secrete GM-CSF.

Analyze results

11. Compare cytokine production by CTL cultures of interest when incubated 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, check to ensure nonspecific CTL lines are not stimulated by B16 to produce cytokine.

To establish CTL lines from any short-term cultures, purify cells by Ficoll gradient and then restimulate CTL on day 6 or 7 after start of secondary culture, as described in Support Protocol 5.

MAINTAINING B16 SPECIFIC CTL CULTURES

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 one 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, the IU values differ among manufacturers. The guidelines mentioned here are for human recombinant IL-2 from Chiron; 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 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 reemerge 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-specific CTL (see Support Protocol 3)

CM/IL-2 (see recipe for complete medium)

6 to 12 week old female C57BL/6 mice

Hanks' balanced salt solution (HBSS; *APPENDIX 2A*)

Complete medium (see recipe), ice cold 10 mg/ml stimulator peptide stock in DMSO (see Table 20.1.1)

60-mm petri dishes

3- or 6-ml syringe

15-ml centrifuge tubes

Disposable cell strainer (Falcon)

Cell irradiator

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3B*) and CO_2 euthanasia (*UNIT 1.8*)

Harvest CTL

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

   If cultures contain a lot of debris, purity by Ficoll gradient. To do this, centrifuge cells for 10 min at 663 × g (in a Sorvall H-2000B rotor at 1500 rpm), room temperature. Count cells and resuspend to roughly 1×10^7 cells/ml in CM. It is important to keep the cells at room temperature. Carefully apply suspension on Lympholyte-M Ficoll, according to manufacturer's recommendations.

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

3. Plate 1 ml CTL (1×10^6 CTL)/well in 24-well plate.

Prepare stimulator cells

4. In laminar flow hood, isolate spleens from naive C57BL/6 mice into a 15-ml centrifuge 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% ethanol to disinfect.

5. Mash spleens thoroughly with sterile butt end of 3- or 6-ml syringe in petri dish with 1 ml ice-cold CM.

   If RBC lysis is desired, transfer spleen to 1 ml ACK lysing buffer (UNIT 3.1) instead of CM, incubate for 2 min at room temperature to allow RBC lysis, and add 9 volumes CM to stop RBC lysis.

6. Transfer splenocytes into 15-ml centrifuge tube and add CM to 10 ml final volume. Pipet vigorously to obtain single-cell suspension.

7. Pass cells through cell strainer into new 15-ml centrifuge tube and centrifuge 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

8. Decant supernatant and resuspend in 10 ml CM. Total cell number will be $\pm 2 \times 10^8$ cells total ($\pm 1 \times 10^8$ per spleen).

Pass through cell strainer if new clumps have formed.

9. Incubate splenocytes 1 hr at 37°C in CM at 1×10^7 cells/ml with 1 to 10 μM peptide.

10. Irradiate splenocytes at 1500 rad to prevent proliferation.

11. Wash splenocytes in 10 ml CM for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C, to remove free peptide. Repeat 2 times for a total of 3 washes.

12. Decant supernatant and resuspend pellet in CM/IL-2 to a final concentration of 3×10^6 cells/ml.

Pass through cell strainer if new clumps have formed.

Add stimulators to CTL

13. Add 1 ml peptide-pulsed stimulator splenocytes (3×10^6 cells)/well to plated CTL (from step 3).

Alternatively, use 3×10^6 unpulsed splenocytes and add 1×10^5 B16 (irradiated 40,000 rad).

14. Culture cells at 37°C, overnight.

Observe daily and split when necessary

15. The following day, replace 1 ml of the medium with 1 ml of fresh CM/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.

MOUSE IMMUNIZATION WITH rVV, rFPV, OR rAd

The construction, amplification, and purification of rVV 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 focolpox 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 sucrose-purified preparations 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; Overwijk et al., 1998; Colella et al., 2000, Bronte 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., 1999).

If necessary, mice can be boosted with virus or another immunogen like 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 boost immunization (Irvine et al., 1997). Spleens or serum can be harvested 2 to 3 weeks after last immunization. This is also the best time to challenge mice with B16; CTL and antibody levels tend to gradually decrease with time.

Materials

Sucrose-purified rVV or rFPV stock at a titer $\geq 1 \times 10^9$ pfu/ml, 1×10^7 pfu/mouse *or* $CsCl_2$-purified rAd at a titer $\geq 1 \times 10^{10}$ pfu/ml, 1×10^9 pfu/mouse

PBS (*APPENDIX 2A*), ice cold

6- to 12-week-old female C57BL/6 mice

Cup sonicator filled with ice water

Heat lamp

Restrainer

3-ml disposable syringes and 27½-G needles

Additional reagents and equipment for mouse restraint *(UNIT 1.3*), ear tagging (*UNIT 1.5*), and intravenous injection of mice (*UNIT 1.6*)

Prepare virus

1. Thaw rVV 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 diluent and should preferably be aliquoted after purification so that no refreezing is necessary.

Immunize mice

4. Relocate work area to 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. Restrain mice using a restrainer that allows tail to protrude. Inject 0.5 ml virus intravenously in tail vein (*UNIT 1.6*) using a 3-ml syringe and 27½-G needle.

Randomize mice

7. If mice have not been pretreated, randomize mice after they have all been injected. Ear tag mice to blind the experiment.

DETECTING ANTIBODIES AGAINST B16 MELANOCYTE DIFFERENTIATION ANTIGENS (MDA) BY IMMUNOBLOT ANALYSIS

It is a good idea to make a large batch of B16 lysate and freeze it in small aliquots of 50 to 100 μl (5 to 10 sample equivalents for immunoblotting) since there can be considerable variation between batches. The B16 lysate also tends to become less reactive with increasing freeze-thaw cycles—i.e., 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, along with the B16 lysate to control for nonspecific staining of other proteins that might be mistaken for MDA.

Instead of B16 melanoma, nonmelanoma cells transfected with plasmid DNA or infected with viral vectors encoding the MDA can be used. However, if Ab 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 Western blot analysis or ELISA (see Support Protocol 8). This protocol directly detects MDA in blotted lysate, and is therefore less sensitive in detecting antibodies than an approach that uses the experimental serum to immunoprecipitate MDA from lysates from metabolically (^{35}S-methionine) labeled B16 cells (Naftzger et al., 1996). However, it is faster and does not require the use of radioactivity.

Materials

B16 culture, ≤50% confluent (see Support Protocol 1)

PBS (*APPENDIX 2A*), ice cold

Lysis buffer (see recipe)

SDS-containing loading buffer (Novex/Invitrogen)

4% to 20% Tris-glycine SDS-PAGE gel (Novex/Invitrogen)

Molecular weight marker (Novex/Invitrogen)

Running buffer with SDS (Novex/Invitrogen)

Transfer buffer (Novex/Invitrogen)

5% (w/v) fat-free powdered milk in PBS

HRP-conjugated anti-mouse antisera (Amersham Pharmacia Biotech)

Secondary antibody from sera of immunized mice (i.e., anti-mouse/HRP)

FAST-DAB substrate with metal enhancer (Sigma-Aldrich)

Disposable cell scraper

Centrifuge and Sorvall H-2000B rotor, 4°C

1-ml disposable syringes and 27 1/2-G needles

Gel apparatus and power source

Nitrocellulose membrane (Novex/Invitrogen)

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 3B*), SDS-PAGE (*UNIT 8.4*), and immunoblotting (*UNIT 8.10*)

Prepare B16 cell lysate

1. Ensure B16 cells are in the logarithmic growth phase when harvested for injection, i.e., flasks should be ≤50% 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. A 50% confluent large flask will yield *~1–5 × 10^7 cells.*

3. Count cells and use 1 × 10^6 cells for each sample lane to be tested.

4. Centrifuge desired number of cells (i.e., 1 × 10^6 per serum sample) for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

5. Carefully remove all supernatant using vacuum suction. Add 10 μl lysis buffer per 1 × 10^6 cells.

6. Add equal volume of SDS containing loading buffer. Boil samples for 2 min.

A beaker containing boiling water can be used. Puncture the cap of vial or tube to allow air to escape.

7. Reduce sample viscosity by shearing sample for 10 to 30 passages through a 1-ml syringe with a 27½-G needle.

Subject B16 lysate to SDS-PAGE

8. Apply lysate on a 4% to 20% Tris-glycine SDS-PAGE gel. Include molecular weight marker in both the first and last gel slot.

Ensure different lysates do not spill over into neighboring slots; if possible, leave empty slots between slots containing different lysates.

9. Run samples for 1 to 2 hr at 125 V and 30 mA, in running buffer with SDS (also see *UNIT 8.4*).

Stop electrophoresis when the bromophenol blue front of the loading buffer has almost reached the bottom of the gel. Most MDA will be located roughly in the middle of the gel.

Blot lysate onto membrane

10. Blot samples onto nitrocellulose membrane for 2 hr at 25 V and 100 mA in transfer buffer (also see *UNIT 8.10*).

Use transfer of marker proteins as a guideline. Ensure that marker proteins under 100 KDa have transferred well from gel to membrane; this ensures that MDA have also transferred.

11. Block nonspecific protein binding by soaking membrane in ample 5% fat-free powdered milk in PBS for 1 hr, room temperature.

Ensure membrane is fully covered with blocking solution.

At this point the membrane can be cut into separate lanes to be probed with different antibodies including positive control, negative control, and experimental samples.

Make sure to clearly label each strip with pencil.

Probe lysate with serum samples

12. Incubate strips for 1 to 2 hr in experimental anti-mouse antisera (e.g., typically 1:100 to 1:2000), room temperature.

13. Wash strips 3 times in 5% fat-free powdered milk in PBS, room temperature.

If necessary, add 0.05% (v/v) Tween-20 to 5% fat-free powdered milk in PBS to reduce background.

14. Incubate 1 to 2 hr in appropriate secondary antibody linked to HRP, usually anti-mouse/HRP, typically 1:1000, room temperature.

15. Wash strips 3 times in 5% fat-free powdered milk in PBS, room temperature.

If necessary, add 0.05% (v/v) Tween 20 to 5% fat-free powdered milk in PBS to reduce background.

Develop staining

16. Incubate strips in FAST-DAB substrate according to manufacturer's protocol. Monitor development and quickly wash away substrate with water when staining is at desired level.

This substrate is simple to use and the procedure fast (i.e., several minutes) but tends to give a lot of background if prolonged exposure is necessary due to weak signal.

Alternatively, incubate with chemiluminescence reagent and expose to X-ray film according to manufacturer's instructions.

This procedure is more sensitive but requires film and a dark room.

DETECTING ANTIBODIES AGAINST MDA BY ELISA

See comments under immunoblot analysis (see Support Protocol 7). Inclusion of negative control cell lysate and negative control serum is of critical importance.

Typical dilutions of sera are 1:10, 1:50, 1:250, 1:1250, 1:6250, 1:31,250. Dilutions are made in PBS with 1% BSA.

Materials

B16 culture, ≤50% confluent (see Support Protocol 1)

PBS (*APPENDIX 2A*), ice cold

1% and 5% (w/v) BSA (Life Technologies) in PBS

Sera from immunized mice

HRP-conjugated anti-mouse antisera (Amersham Pharmacia Biotech)

o-phenylenediamine dihydrochloride (OPD; Sigma)

4 M H_2SO_4

Disposable cell scraper

Sorvall H-2000B rotor, 4°C

PVC microtiter plates (Dynex Technologies)

ELISA reader

Prepare B16 cell lysate

1. Ensure that B16 cells are in the logarithmic growth phase when harvested for injection, i.e., flasks should be ≤50% 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. A 50% confluent large flask will yield *~1–5 × 10^7 cells.*

3. Count cells (*APPENDIX 3B*). Dispense 1 × 10^4 cells per well.

4. Centrifuge desired number of cells (i.e., 1 × 10^4 per well) for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

5. Carefully remove all supernatant using vacuum suction. Resuspend cells at 2 × 10^5 cells/ml in PBS, transfer to freeze-resistant tubes, and lyse by 3 freeze/thaw-cycles.

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

Coat and block plates

6. Plate lysate at 1 × 10^4 cell equivalents/well (e.g., 50 μl) into PVC microtiter plates. Dry overnight at 37°C.

7. 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

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

Typical dilutions used are 1:10, 1:50, 1:250, 1:1250, 1:6250, and 1:31,250.

9. Wash wells three times with 1% BSA in PBS, room temperature.

10. Incubate with HRP-conjugated anti-mouse antisera, typically 1:4000, in 1% BSA in PBS for 1 hr, 4°C.

11. Wash three times with 1% BSA in PBS, room temperature.

Develop staining

12. Develop color with OPD according to manufacturer's procedures. Add 50 μl OPD substrate/well and incubate 10 min, room temperature.

13. Stop reaction by adding 50 μl 4 M H_2SO_4 per well.

14. Read plates at 492 nm in an ELISA reader.

REAGENTS AND SOLUTIONS

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

Complete medium (CM)

10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)

1 mM sodium pyruvate (final concentration)

2 mM L-glutamine

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/ml rhIL-2 (e.g., equivalent to 5 to 10 Chiron units/ml)

Store up to 2 weeks in the dark at 4°C.

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

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.*

Lysis buffer

PBS (*APPENDIX 2A*) containing:

0.02% (v/v) Triton X-100

1% (w/v) aprotinin

Store up to 1 year, 4°C

COMMENTARY

Background Information

B16 melanoma is a spontaneous melanoma derived from C57BL/6 mice. As a model for human tumors, it is important to note similarities and differences between B16 and human melanomas (see Table 20.1.2). Several variants were isolated by Dr. Isaiah J. Fidler in the mid-1970s, the main differences between sublines 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 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; E. Gorelik, pers. comm.).

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. It tends to be one of the tougher tests for any form of immunotherapy.

How to induce immune responses: 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 to 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), vaccination with baculovirus encoding mTRP-1 (Naftzger et al., 1996), or 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 retroviral antigen p15E (Bloom et al., 1997; Overwijk et al., 1998; Zeh 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., 1999).
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., 1999).

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 towards target antigens, for example, by culturing $CD4^+$ and $CD8^+$ T lymphocytes with specific antigen and subsequent 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 7). 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, β_2m, TAP-1, RAG-1, or Igh KO mice) available from specialized breeding companies such as Jackson Laboratories and Taconic Farms.

Discrepancies between results from in vivo and in vitro assays—Adoptive transfer of MDA-specific CTL can result in dramatic reductions in pulmonary tumor burden. In addition, vaccination with rVVhgp100 induces CTL that are cross-reactive with mgp100, and recognize B16 upon in vitro stimulation with $mgp100_{25\text{-}33}$ peptide. Still, mice vaccinated with rVVhgp100 are not protected from B16 tumor challenge. This may be due to the low MHC Class I expression of B16, rendering it susceptible only to killing by large numbers of in vitro–activated CTL. It is possible that combination of recombinant vaccines with additions such as cytokines, B7-1 costimulatory molecules, or anti-CTLA4 antibody will result in CTL responses powerful enough to impact B16 growth. Currently, the most convincing data available on the correlation between in vitro assays and in vivo anti-tumor effects comes from tumor models in which tumor cells express defined foreign antigens such as β-galactosidase or influenza NP. These models suggest that vaccine-induced tumor regression is usually dependent on $CD8^+$ CTL, and sometimes also on $CD4^+$ T cells. Vaccine strategies that induce the most powerful CTL typically also have the strongest anti-tumor effect. CTL responses are typically measured in killing assays such as ^{51}Cr-release assays or cytokine release assays by ELISA (see Support Protocol 4).

Critical Parameters

One of the most important aspects of in vivo mouse experiments is ensuring that tumor taking among mice is highly consistent. Inconsistent injection techniques make it very difficult to distinguish therapeutic effects over random fluctuations in tumor take. Practicing in 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 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 20.1.3 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 pulmonary metastasis treatment experiment lasts 16 to 18 days from the day of tumor inoculation. Sufficient amounts of tumor cells and CTL should be ready for use on the appropriate days (usually day 3 after tumor inoculation); in addition CTL for adoptive transfer should be used between 5 and 10 days after last restimulation. Subcutaneous tumors can be allowed to grow for 2 to 3 weeks before mice must be sacrificed due to large tumor burden. To induce vitiligo, two injections of rVVmTRP-1 are spaced at least 2 weeks apart, upon 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.

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Table 20.1.1

Human and Mouse Melanoma/Melanocyte Differentiation Antigens Recognized by Melanoma-Reactive T Cells

Gene	Restriction element	Peptide epitope[a]	Reference
gp100/pmel17	HLA-A*0201	KTWGQYWQV	Kawakami et al., 1995; Cox et al., 1994
		AMLGTHTMEV	Tsai et al., 1997
		MLGTHTMEV	Tsai et al., 1997
		ITDQVPFSV	Kawakami et al., 1995
		YLEPGPVTA	Kawakami et al., 1995; Tsai et al., 1997
		LLDGTATLRL	Kawakami et al., 1995
		VLYRYGSFSV	Kawakami et al., 1995
		SLADTNSLAV	Tsai et al., 1997
		RLMKQDFSV	Kawakami et al., 1998
		RLPRIFCSC	Kawakami et al., 1998
	HLA-A3	ALLAVGATK	Skipper et al., 1996
		LIYRRRLMK	Kawakami et al., 1998
	HLA-A*2402	VYFFLPDHL	Robbins et al., 1997
	HLA-DRB1 *0401	WNRQLYPEWTEAQRLD	Touloukian et al., 2000
	H-2D^b	EGSRNQDWL	Overwijk et al., 1998
		KVPRNQDWL	
Tyrosinase	HLA-A*0201	MLLAVCYLL	Wolfel et al., 1994
		YMDGTMSQV	Wolfel et al., 1994
	HLA-A1	DAEKCDKTDEY	Kittlesen et al., 1998
		SSDYVIPIGTY	Kawakami et al., 1998
	HLA-A*2402	AFLPWHRLF	Kang et al., 1995
	HLA-A*0201/ H-2D^d	FMDGTMSQV	Colella et al., 2000
		YMDGTMSQV	
	HLA–B44	SEIWRDIDF	Brichard et al., 1996
	HLA-DR4	QNILLSNAPLGPQFP	Topalian et al., 1996
		SYLQDSPDSFQD	
MART-1/ Melan-A	HLA-A*0201	AAGIGILTV	Kawakami et al., 1994
	HLA-B*4501	AEEAAGIGILT	Schneider et al., 1998
TRP-1/gp75	HLA-31	MSLQRQFLR	Wang et al., 1996b
	H-2 K^b	TWHRYHLL	Dyall et al., 1998
		TAYRYHLL	
TRP-2	HLA-A*0201	SVYDFFVWL	Parkhurst et al., 1998
	HLA-A31	LLPGGRPYR	Wang et al., 1996a
	HLA-A33	LLPGGRPYR	Wang et al., 1996a
	H-2K^b	SVYDFFVWL	Bloom et al., 1997

[a]Peptides of 15 amino acids or less can be synthesized without much difficulty on commercially available peptide synthesizers, though it is recommended that each peptide be tested for purity by mass spectrometry. Both synthesis and analysis can be contracted out to many companies. Peptide stocks are prepared by dissolving the weighed powder in 100% dimethylsufoxide (DMSO), usually at a concentration of 10 mg/ml, and stored at 4°C in air-tight vials or tubes (DMSO is hygroscopic and a solid at 4°C).

Table 20.1.2

B16 as a Model for Human Melanoma: Similarities and Differences

Similarities[a]	Differences
Human melanomas express at least 5 different MDA: gp100/pmel17, 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 biopsies in IL-2 will yield melanoma-specific CTL in ~50% of cases; B16 biopsies grown in IL-2 rarely yield B16 specific CTL.
Vitiligo is correlated with favorable clinical prognosis in human melanoma patients; mice with rVVmTRP-1-induced vitiligo are protected from B16 tumor challenge.	Adoptive transfer of CTL (TIL) can reduce subcutaneous melanoma burden in humans; adoptive transfer of gp100-specific CTL does not significantly impact on subcutaneous B16.
Adoptive transfer of gp100-specific CTL can reduce tumor burden in humans; adoptive transfer of gp100-specific CTL can reduce pulmonary B16 metastases in mice.	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.
Human melanomas can often be induced to express MHC Class II by IFN-γ treatment; B16 expresses MHC Class II upon IFN-γ treatment (Bohm et al., 1998, D.R. Surman, pers. comm.).	

[a] In conclusion, B16 melanoma is a reasonable model for human melanoma. If anything, its rapid growth, low MHC Class I expression, and its unresponsiveness to adoptive CTL treatment of subcutaneous disease compare unfavorably with most human melanomas and suggest treatment of B16 is a rigorous test for immunotherapy of murine cancer.

Table 20.1.3

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
	Overtrypsinization 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	Keep tumor on ice at all time
	Injected too few tumor cells	Increase inoculum of tumor cells
Poor treatment upon adoptive transfer of CTL	Low potency or specificity of CTL	Test CTL in vitro directly prior to transfer to ensure specificity
	Too few CTL transferred	Transfer more CTL, or administer IL-2
Poor CTL growth	Too little IL-2 in cultures	Prepare new IL-2 stock, check type of units
	Poor FBS quality	Test different FBS lots
	“Dirty” cultures	Purify on Ficoll to eliminate debris
	Over- or understimulation	Restimulate every 7-10 days
	Overcrowding of culture wells	Split once CTL exceed 50% confluency
Poor CTL specificity/high background (often irreversible)	Too much IL-2 in cultures	Keep [IL-2] between 30-60 IU/ml
	“Dirty cultures”	Ficoll 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	Retiter viral stock
	Mixup of rVV stocks	Check expression by immunostaining of infected cells