

Particle-Mediated Gene Transfer into Dendritic Cells

A Novel Strategy for the Induction of Immune Responses against Tumor Antigens

Thomas Tüting and Andreas Albers

1. Introduction

The expression of a foreign protein in the skin following direct in vivo gene transfer results in the induction of potent cellular and humoral immune responses. This strategy, now known as genetic or DNA immunization, was first described by Johnston et al. in 1992. They reported that bombardment of murine skin with an expression plasmid encoding human growth hormone coated onto microscopic gold particles using a gene gun resulted not only in the systemic delivery of the molecule, but also in the induction of antigen-specific antibody responses (*1*). It is now well established that DNA immunization by particle-mediated gene transfer promotes broad-based and long-lasting antigen-specific immune responses capable of protecting against challenges with infectious agents and tumor cells in rodents [reviewed in (*2,3*)]. Importantly, gene gun immunization elicits both humoral and cellular immunity, consisting of antibody responses specific for conformational determinants, as well as, antigen-specific CD8⁺ cytotoxic T cells and CD4⁺ T-helper cells. For this reason, it represents an attractive novel approach for the clinical development of prophylactic and therapeutic vaccines against certain infectious diseases and tumors. We are currently investigating, like many other tumor immunologists, whether DNA-based immunization can lead to the induction of potent cellular and humoral immune responses against defined tumor antigens that are associated with tumor rejection activity in vivo.

1.1. Dendritic Cells are Critical for the Induction of Immune Responses Following Cutaneous Gene Gun Immunization

The molecular mechanisms responsible for the antigen-specific stimulation of naive lymphocytes following transgenic expression of foreign proteins in the skin has been poorly understood until recently. Studies using bone marrow chimeric mice (4–7) have demonstrated that cellular responses to plasmid-encoded antigens are dependent upon bone marrow-derived cells rather than on keratinocytes, which are the predominantly transfected cell type after direct *in vivo* gene transfer to the skin. These observations were consistent with emerging evidence in the field of immunology, suggesting a pivotal role for the dendritic cell (DC) system of antigen presenting cells (APC) for the induction of cellular immune responses [reviewed in (8,9)]. DCs are bone marrow-derived leukocytes that are specialized for antigen capture, processing, and presentation. They are frequently found in the skin and mucous membranes where they function as sentinels of the immune system. Activation of DC in response to inflammatory stimuli followed by antigen capture, functional maturation, and subsequent migration appears to be a key event in initiating immunity. Activated, mature DC localize to T-cell rich areas of secondary lymphoid organs where they display high levels of processed antigen in association with MHC molecules on their cell surface. Most importantly, activated DC produce chemotactic factors for naive T cells and provide the antigen in the context of secondary immunostimulatory signals (costimulatory molecules like B7.1/CD80, B7.2/CD86, and CD40) and immunostimulatory cytokines (like TNF- α , IL-6, and IL-12), which promote the activation and expansion of antigen-specific T cells. Evidence has now accumulated that particle-mediated gene transfer to the skin leads to direct *in vivo* transfection of cutaneous DC associated with endogenous antigen synthesis and processing (10). Particle bombardment itself appears to represent an activating stimulus for the DC system leading to DC migration to regional lymph nodes, where antigen-expressing DC can efficiently stimulate proliferation of antigen-specific CD8⁺ as well as CD4⁺ T lymphocytes (11,12). DC migrating out of skin injected with plasmid DNA can stimulate antigen-specific cellular immune responses *in vitro* and *in vivo* when transferred to naive mice (11–14). Advances in cell-culture technology now allow for the *in vitro* generation of immunostimulatory dendritic cells from precursors in bone marrow or peripheral blood (15–16). Cultured DC, gene gun-transfected *in vitro* with antigen-encoding plasmid DNA, are also capable of stimulating antigen-specific immune responses *in vitro* and, following their adoptive transfer, *in vivo* (17–19). The possibility to immunize with cultured DC genetically engineered to endogenously express a given

antigen represents an exciting new method both for basic science and clinical applications.

1.2. The Immune Response Can Be Manipulated at the Molecular Level

The use of plasmid DNA as a source of antigen for gene gun immunization opens up a number of completely new strategies allowing researchers to investigate the regulation of adoptive immunity and try to deliberately influence the nature of immune responses. This can be achieved by simultaneous delivery of plasmids encoding immunomodulatory proteins, revealing their role in the recruitment, antigen-capture, activation, and migration of DC, as well as, in the stimulation of responding T and B cells. As an example, codelivery of plasmid DNA encoding the costimulatory molecules B7 and CD40L and the cytokines GM-CSF, IFN- α , or IL-12 have been shown to enhance the induction of cellular immune responses following DNA immunization in mice (20–24). These factors are known to stimulate growth and maturation of DC and influence the expansion and phenotype of responding T cells. Another attractive idea is the possibility to selectively target antigen into the processing machinery of immunostimulatory DC for effective presentation to both CD8⁺ and CD4⁺ T lymphocytes. This is currently being attempted by translational targeting of antigen expression in dendritic cells in vivo using a DC-specific promoter element or by transfection and adoptive transfer of cultured DC. Furthermore, genetic reengineering of the actual antigen-encoding cDNA allows for targeting antigen into MHC Class I and II loading compartments by manipulation of signals governing intracellular protein transport (25,26).

1.3. Gene Gun Immunization Can Be Applied for the Induction of Immune Responses Against Defined Tumor Antigens

With the molecular identification of tumor antigens (27,28), there has been increasing interest in DNA immunization for the development of active specific tumor vaccines. It is envisioned that such strategies may be clinically applied in an adjuvant setting to cancer patients who are macroscopically tumor-free following successful surgery. It is hypothesized that the induction of a strong antigen-specific immune response can eliminate residual tumor cells resulting in a clinical benefit by reducing the risk of recurrence. We and others have shown that DNA immunization of mice with model tumor antigens such as chicken ovalbumin or β -galactosidase (10,21–23) induced protective immune responses leading to partial rejection of a subsequent, normally lethal challenge with tumor cells expressing the respective model antigen. More



Fig. 1. Helios Gene Gun.

recently, DNA immunization has also been shown effective in murine models employing clinically relevant tumor antigens. Tumor rejection activity was observed following immunization with a mutated form of the transformation-related antigen p53 (22), with the idiotypic determinant of the immunoglobulin expressed on the surface of a B-cell lymphoma (29), with the tumor-specific “cancer-testes” antigen P815 (30), and with melanocyte differentiation antigens such as gp100, TRP-1, and TRP-2 (31–33). Studies are underway in a large number of laboratories to characterize the antitumor immune responses induced following DNA immunization in greater detail. Careful comparisons with established vaccination methods need to be performed. Furthermore, strategies for immunization need to be optimized, for example by targeting tumor antigen-expression to DC. Eventually, clinical trials will have to be conducted in cancer patients. The early results of clinical trials involving DNA immunization for the prevention and treatment of infectious diseases appear to be promising (34–37).

1.4. Particle-Mediated Gene Transfer Directly Delivers Plasmid DNA to the Nucleus

The general principles of particle-mediated gene transfer have been detailed by Rakhmievich and Yang in Chapter 20. Briefly, plasmid DNA is precipitated onto 0.6–3 μm gold beads, which are subsequently loaded into pieces of plastic tubing as a cartridge, and placed in the gene gun (Fig. 1). A short helium pulse accelerates the plasmid-coated gold beads towards the target



Fig. 2. Biolistic PDS/1000He with sevenfold pressure distribute.

tissue, resulting in efficient intracellular delivery of high copy numbers of DNA *in vivo*. Particle-mediated gene transfer has recently been technologically advanced for effective *ex vivo* gene delivery into cells in culture. A vacuum chamber is used for gentle acceleration of gold beads. Additionally, the ballistic approach is combined with magnetic separation of transfected cells. This system is now called ballistomagnetic vector system (BMVS) (**Fig. 2**). Gold particles are simultaneously coated with nucleic acids and superparamagnetic beads and propelled into target cells. It can be shown that plasmid DNA and magnetic beads penetrate plasma membranes and other cellular barriers and are directly delivered into the nucleus of mammalian cells. This is of particular importance for expression of transgenes because plasmid DNA can only become transcriptionally active if it reaches the nucleoplasm. Superparamagnetic beads, simultaneously delivered with the nucleic acids, allow for efficient separation of transfected and nontransfected cells in a high gradient magnetic field (Schroff et al., manuscript submitted). A new procedure of plating cells on microporous polycarbonate supports has been developed to improve gene transfer efficiency, as well as, recovery and viability of cells. In this way, subsequent enrichment of the ballistomagnetically transfected cell population via high gradient magnetic separation columns becomes much more efficient (Albers et al., manuscript in preparation).

1.5. Practical Considerations

We describe here the use of particle-mediated gene transfer for the induction of immune responses against (tumor) antigens in murine tumor models. We recommend that interested researchers begin their experiments with marker genes such as β -galactosidase and EGFP, which can be readily detected and can simultaneously serve as model tumor antigens. We have found β -galactosidase very convenient in mice, since several CTL-defined peptide epitopes have been identified for both BALB/c and C57BL/6 strains and β -galactosidase is available as a recombinant protein. Mice can be immunized by directly bombarding the skin with plasmid DNA. Here we describe the preparation of cartridges with DNA-coated gold beads and the *in vivo* gene transfer into skin using the Helios Gene Gun. We also describe protocols for the measurement of humoral and cellular immune responses using β -galactosidase in BALB/c mice. These protocols can subsequently be adopted to other antigens.

Alternatively to direct *in vivo* immunization with the Helios Gene Gun, mice can be immunized by injection of genetically modified immunostimulatory dendritic cells. Cultured DC can be transfected with the hand held gene gun. Transfected DC can subsequently be used to immunize mice and immune responses monitored. However, we believe that the ballistomagnetic transfer using the Biolistic PDS/1000He system modified by a sevenfold pressure distributor system ensuring particle distribution over the entire Petri dish will be much more suitable for efficient and reproducible gene transfer to cultured DC *in vitro*. This system has optimized physical parameters for *in vitro* particle-mediated gene delivery and allows simultaneous transfection of large numbers of cells. We are currently evaluating this system for gene transfer to cultured DC. Gold particles (usually 1.6 μm in diameter) are coated with a mixture of plasmid DNA and much smaller superparamagnetic particles (65 nm in diameter). Following ballistomagnetic gene transfer, cells are immediately washed, resuspended, and transferred onto a high gradient magnetic separation column to enrich for cells containing paramagnetic beads (and DNA). This procedure ensures that >90% of living cells contains DNA, a subset of which will express the gene (or genes) of interest. The ballistomagnetic vector system has been established for the production of genetically modified tumor cell vaccines under GMP conditions at the Centrum Somatische Genterapie, Berlin, Germany. It has proven to be highly reproducible and can readily be quality controlled and, therefore, appears ideally suited for possible future clinical applications regarding genetic immunization with tumor antigen-transfected DC.

2. Materials

2.1. Mice, Cell Lines, Media, and Cytokines

1. Female C57BL/6 (H-2^b) and BALB/cJ (H-2^d) mice, 6–10 wks old. We have purchased from any of the large vendors (i.e., Charles Rivers, Jackson Laboratory, or Taconics).
2. EL4 is a murine thymoma available from the American Type Culture Collection (Rockville, MD). EL4-transfectants expressing β -galactosidase, EGFP or hgp100 have been generated from various laboratories. C3 is an HPV 16-transformed murine sarcoma (38). C26 is a chemically induced BALB/c murine adenocarcinoma (39).
3. Our cell culture medium (CM) consists of RPMI-1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM HEPES, 50 μ M 2-ME, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. All cell culture reagents can be purchased from Life Technologies.
4. Recombinant murine IL-4, murine GM-CSF, and human IL-2. We use cytokines given to us from Schering-Plough Research Institute (New Jersey) and Chiron (Emeryville, CA). They can be bought from commercial suppliers such as Genzyme as well. Cytokines are diluted to 10⁶ U/mL of complete medium and stored frozen at –80°C. Once thawed, they can be stored at 4°C and should be used within 4 wks.

2.2. Plasmid DNA, Synthetic Peptides, β -Galactosidase Detection

1. Plasmids encoding reporter genes or tumor antigens: pCI- β gal, pCI-EGFP, pCI-E7, pCI-hgp100. These plasmids were constructed by inserting fragments containing the *Escherichia coli* β gal gene, the EGFP gene (Clontech, Palo Alto, CA), the ORF of human papilloma virus (HPV) 16-E7, or the cDNA encoding the melanosomal protein gp100/pm17 into the expression vector pCI (Promega, Madison, WI). We grow plasmids in *E. coli* strain DH5 α , use Qiagen Endofree Plasmid Maxi Kits (Qiagen, Chatsworth, CA) for purification, resuspend in distilled water, and store plasmids at –20° (see **Note 1**).
2. CTL-defined synthetic peptides: We have used the H-2K^b-binding β gal-encoded peptide DAPIYTNV (β gal_{96–103}), the H-2L^d-binding β gal-encoded peptide TPHPARIGL (β gal_{876–884}), the H-2D^b-binding HPV-E7-encoded peptide RAHYNIVTF (E7_{49–57}), and the H-2D^b-binding hgp100-encoded peptide KVPRNQDWL (hgp100_{25–33}). These peptides were synthesized commercially by standard F-moc chemistry and purified by HPLC. Peptides are diluted at 10 mg/mL in PBS containing various amounts of DMSO and stored frozen at –20°C (see **Note 2**).
3. Reagents for detection of β -galactosidase: X-gal substrate (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, Sigma Chemical Co., St. Louis, MO) dissolved

in dimethyl formamide at 20 mg/mL (can be stored frozen at -20°C). Mix prior to use 1:25 (40 $\mu\text{L}/\text{mL}$) with buffer solution containing 44 mM HEPES buffer, 3.1 mM K^+ ferricyanide, 3.1 mM K^+ ferrocyanide, 150 mM NaCl, 10 mM Na_2HPO_4 , 10 mM Na_2HPO_4 , 1 mM MgCl_2 , pH 7.4 mL (can also be stored frozen at -20°C).

2.3. DC Culture and Phenotyping

1. 70% EtOH, scalpel, scissors, forceps, 10 cm Petri dishes, 18G and 23G needles, 10/20 mL syringes, 50 mL polypropylene tubes, 6-well plates, cell strainer (70 μm nylon, Falcon 2350) or sterile nylon mesh (*see Note 3*).
2. RBC lysing buffer (Sigma Chemical Co.) or self-made: Dissolve 8.29 g NH_4Cl in 700 mL dH_2O on stirrer, add 1 g KHCO_3 , and finally 0.0372 g of Na_2EDTA . Fill up to 1 L, filter-sterilize.
3. Hybridoma supernatants (optional): anti-mCD4 (L3T4, ATCC TIB 207); anti-mCD8 (Lyt 2.2, ATCC TIB 210); anti-B220 (ATCC TIB 146).
4. Antibodies for FACS staining: Isotype controls, anti-IA^b or -IA^d, anti-CD40, anti-B7.1 (CD80), anti-B7.2 (CD86), and anti-CD11c, anti-B220, anti-CD3, anti-GR-1, and anti-NK1.1. All antibodies are available from Pharmingen (*see Note 4*).

2.4. Handheld Gene Gun

1. The Helios Gene Gun System is now available from Bio-Rad (Richmond, CA) including helium regulator, tubing prep station, and tubing cutter.
2. Helium gas 4.6, personal hearing protection.
3. Gold beads: Bio-Rad sells 1.6 μm gold beads. We have also successfully used 1–3 μm beads purchased from Strem Chemicals (*see Note 5*).
4. Tefzel Tubing.
5. Analytical balance, microfuge, ultrasonic cleaner bath, hair clippers.
6. 15 mL and 50 mL polypropylene tubes, 1.5 mL microfuge tubes.
7. Spermidine, CaCl_2 , 100% (dry) ethanol, polyvinylpyrrolidone PVP (Sigma Chemical Co.).

2.5. Ballistomagnetic Vector System (BMVS)

1. The Biolistic PDS 1000/He System for particle mediated gene delivery including the Hydra-Adaptor and the membrane vacuum pump Vaccubrand MZ 2C is available from Bio-Rad.
2. Gold beads/microcarrier: Bio-Rad sells good quality beads (\varnothing 0.6 μm , 1.0 μm , and 1.6 μm).
3. Stopping-screens.
4. Macrocarrier.
5. Rupture discs 450 psi–2200 psi.
6. Superparamagnetic beads, separation columns, and the magnet are available from Miltenyi Biotec.
7. Superparamagnetic beads: Basic MicroBeads.
8. Separation columns: Depletion Column Type AS.

9. Magnetic cell separator: VarioMACS.
10. Helium gas 4.6.
11. PBS without Ca^{2+} and Mg^{2+} (Biowhittaker).

2.6. Detection of Immune Responses

1. ELISA-Reader, dissecting microscope, gamma counter.
2. ELISA plates (we use Nunc Maxisorp).
3. Recombinant β -galactosidase protein (Sigma).
4. Peroxidase-conjugated goat antimouse IgG Ab (Sigma), isotype-specific goat anti-mouse IgG₁ and IgG_{2a} Ab, peroxidase-conjugated donkey antigoat IgG Ab (Jackson Research Lab.), We use TMB peroxidase Substrate System (Kierkegaard and Perry) for detection.
5. Millipore HA ELISPOT plates.
6. Antibodies to mouse IFN- γ : purified antimouse IFN- γ (clone R4-6A2, Pharmingen) for capture and biotinylated rat antimouse IFN- γ (clone XMG1.2, Pharmingen) for detection; streptavidin-peroxidase conjugate (Boehringer Mannheim, Mannheim, Germany); peroxidase substrate kit DAB (Vector Labs., Burlingame, CA).
7. BSA fraction V, Tween 20, PBS, Thimerosal (Sigma).
8. Na_2CO_3 , NaHCO_3 , NaCl , $\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$.
9. 24-well and 96-well round-bottom plates.
10. $\text{Na}_2^{51}\text{CrO}_4$ (NEN-Dupont, Bedford, MA).
11. Titer tubes (Bio-Rad).

3. Methods

3.1. Gene Transfer Directly to Skin In Vivo

3.1.1. Coating DNA on Gold Particles

1. We routinely use 0.5-in cartridges containing 1 μg DNA coated onto 0.5 mg gold beads. In each preparation we aim for 50 cartridges and need 25 mg gold and 50 μg DNA.
2. Make a 0.05M Spermidine solution and a 1M CaCl_2 solution. Dissolve PVP at 1 mg/mL in 100% ethanol and seal with parafilm. All solutions can be stored frozen at -20°C .
3. Weigh 25 mg gold for 25 inches of tubing (= 50 bullets) in a 1.5-mL microcentrifuge tube.
4. Resuspend 25 mg gold in 100 μL 0.05M spermidine, vortex and sonicate briefly in the ultrasound cleaner bath to disrupt clumps. Add 50 μg of plasmid DNA in 50 μL water and vortex. Plasmid DNA should be at 1 $\mu\text{g}/\text{mL}$ in distilled water.
5. While vortexing, slowly add 100 μL of 1M CaCl_2 dropwise to precipitate DNA onto gold beads. Allow gold beads to settle for 10 min (*see Note 6*).
6. Centrifuge beads and remove supernatant. Disrupt pellet and wash three times with 100% ethanol to remove H_2O . Resuspend gold beads in 200 μL of 100%

ethanol containing 0.075 mg/mL of PVP and transfer to a 15-mL polypropylene tube. Add 3 mL of 100% ethanol containing 0.075 mg/mL. The DNA-coated bead suspension can be stored several weeks at -20°C . Seal tubes with parafilm before freezing.

3.1.2. Loading DNA-Coated Gold Particles into Tefzel Tubing

1. Set up tubing prep station and connect to nitrogen tank. Flush tubing 15 min with nitrogen prior to loading with beads to dry completely (*see Note 7*).
2. Vortex and sonicate the ethanol solution containing the DNA-coated gold beads. Immediately draw the solution into the tubing using the syringe and load into the tubing prep station.
3. Allow beads to settle for 3–5 min.
4. Remove ethanol at 0.5–1.0 in/s from the Tefzel tubing, quickly turn the tubing by hand, and start to rotate the tubing to distribute the gold beads evenly.
5. After 30 s, turn on the nitrogen flow (0.4 LPM) and dry gold beads inside the tubing for about 5–10 min (*see Note 8*).
6. Cut tubing in bullets 0.5-m inches long. Store in 50 mL polypropylene tubes along with dessiccant pellets. Seal with parafilm and store bullets at 4°C . Bullets are stable for several months (*see Note 9*).

3.1.3. Gene Gun Immunization of Mice

1. Shave the abdominal skin of the mice and label the cages. Load the cartridges into the cartridge holder. Put on hearing protection.
2. Prepare the gene gun and test its function with an empty cartridge holder, setting the discharge pressure to 400 psi (*see Note 10*).
3. Load gene gun with full cartridge holder and immunize mice.
4. We routinely immunize by bombarding the abdomen. In this way, one can conveniently hold the mouse and shoot without anaesthesia. We usually shoot twice, resulting in the delivery of 2 μg of plasmid DNA for each immunizations.
5. You can sacrifice mice 24–36 h following particle-mediated gene transfer, depilate and harvest bombarded skin, snap freeze in liquid nitrogen, and store at -80° . Cryosections can be fixed in 0.5% glutaraldehyde, stained for β -gal expression with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, Sigma Chemical Co.) 1 h at room temperature, counterstained with hematoxylin, and analyzed. EGFP expression can be directly visualized under a fluorescent microscope.

3.2. Gene Transfer to DC In Vitro and Adoptive Transfer

3.2.1. Culture of DC

1. Sacrifice mouse immediately before harvest. Clean mouse with 70% EtOH. Make a transverse incision across belly and deskin mouse to expose the entire hind legs.
2. Using scalpel or fine sterile scissors, remove femur and tibia bilaterally, taking care to remove as much surrounding muscle as possible (bone does not need to be completely clean). Place bones in Petri dish on ice.

3. Put 3 Petri dishes under hood, one with 70% EtOH, two with RPMI-1640 (no serum). Place bones for 2 min in alcohol, then wash in RPMI-1640 and transfer to third dish with RPMI-1640.
4. Taking the femur, cut off the epiphyseal plates at both ends of the bone (area of thickening) until the red bone marrow is visible centrally. Using a 23G needle attached to a 10/20 mL syringe, squirt 1–2 cc RPMI-1640 through the bone marrow canal (from top to bottom) into the Petri dish. You should see the red marrow contents exit the bone. Harvest the bone marrow from the tibia similarly.
5. Disrupt cell clusters by vigorous pipeting and filter through a cell strainer (70 μ m nylon, Falcon 2350) or sterile nylon mesh into a 50 mL tube to remove all debris.
6. Centrifuge at 600g for 5 min at RT. Decant supernatant.
7. Resuspend the pellet in 1 mL/per mouse of red blood cell lysing buffer, incubate at RT for 2 min. Neutralize with 10–20 mL complete medium.
8. Centrifuge cells again and resuspend pellet in 10–20 mL complete medium and count. Yield should be approximately 25×10^6 cells/mouse. Dilute and distribute cells into 6-well (we used Costar or Falcon plates) at 10^6 cells/3 mL medium containing 500 U/mL mGM-CSF + 500 U/mL IL-4/well (see **Notes 11** and **12**).
9. After 48 h, remove 2 mL of the medium after *gentle* swirling of the plate. This removes many of the contaminant B cells, granulocytes, and NK cells. Care must be taken not to disrupt the DC clusters that are loosely adherent. Add 2 mL of fresh cytokine-containing medium.
10. After 4 and 6 d, fresh cytokine-containing medium should be added/replaced (2 mL/well). After 7–8 d, you will have about 20×10^6 cells per mouse with approximately 60–80% of total cells exhibiting DC morphology and phenotype (MHC II⁺, CD40⁺, B7.1⁺, B7.2⁺, CD11c⁺, B220⁻, Gr1⁻, CD3⁻, NK1.1⁻) (see **Note 13**).
11. This system has also been developed using 1.5% freshly harvested autologous mouse serum (MS) instead of FBS in the culture medium. If MS is used, yields are reduced significantly on day 7–8 harvests.

3.2.2. In Vitro Gene Transfer to DC Using the Hand-Held Gene Gun

1. Harvest murine bone marrow-derived DC, count, and centrifuge. Meanwhile, prewet 6-well plates with HEPES-buffered complete medium and prepare the gun.
2. Carefully resuspend DC in HEPES-buffered complete medium at 2×10^6 cells/25 μ L per transfection and place on ice. Completely remove medium from 6-well plates, leaving only a film that will allow the DC suspension to spread evenly.
3. Place 2×10^6 DC/25 μ L in the center of the well, shoot from optimal distance as recommended by the manufacturer, and immediately add CM. We have bombarded at a pressure range of 250–300 psi of helium with the Accell device using a “spinner,” which is placed in front of the cylinder and rotates the helium flow to give an even distribution of gold beads over the target range (see **Note 14**).

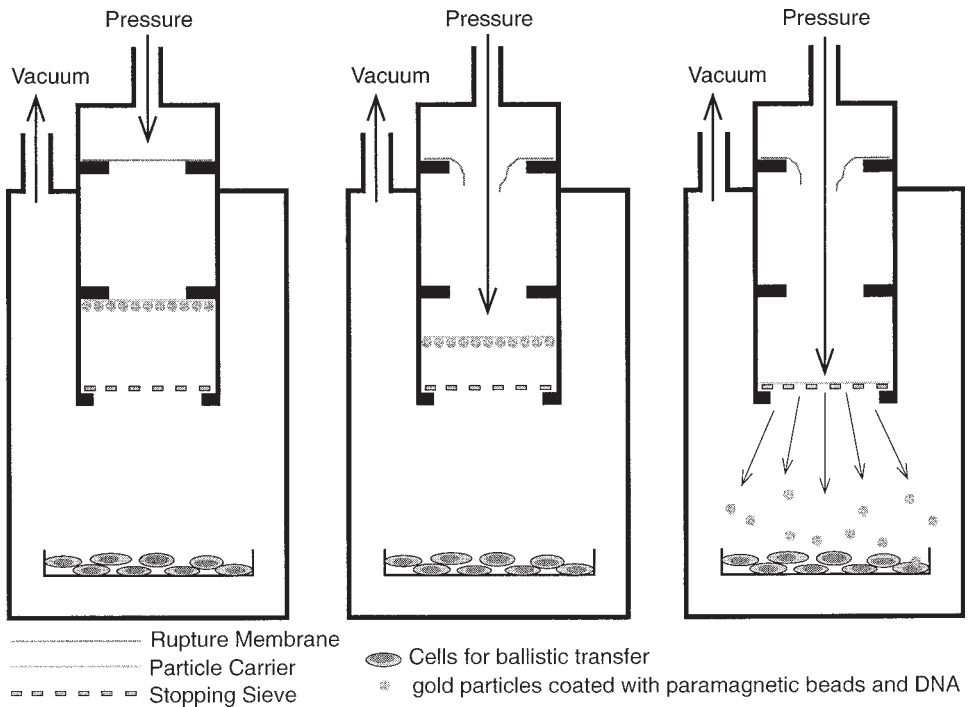


Fig. 3. Operation of the Biolistic Device for in vitro gene transfer to cell suspension.

3.2.3. In Vitro Gene Transfer Using the Ballistomagnetic Vector System (BMVS) Operation of the Biolistic Device (Fig. 3)

1. Pipet 15 μL of a colloidal gold suspension (60 mg Au/mL H_2O) in the center of seven macrocarrier, let the gold particles sediment, remove carefully excess supernatant water (see **Note 15**).
2. Resuspend the gold particles in 30 μL of a mixture of three parts of DNA (1 $\mu\text{g}/\mu\text{L}$) and one part superparamagnetic bead solution (see **Note 16**).
3. Let the gold particles sediment again, remove the supernatant, let the gold particles dry (see **Note 17**).
4. Load the seven macrocarrier into the launch assembly and mount the pressure distributor and the launch assembly into the Biolistic PDS/1000He system.
5. Remove all supernatant from the cells to be transfected.
6. Place the Petri dish containing the cells into transfection chamber.
7. Draw vacuum to 20 in Hg and operate the Biolistic PDS/1000He.
8. Resuspend the cells immediately after transfection in PBS containing 1% FCS and 2 mM EDTA.

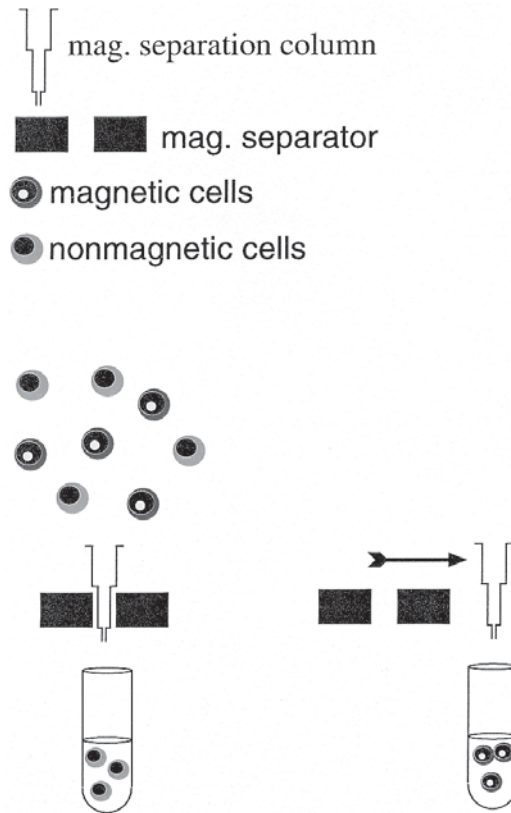


Fig. 4. Magnetic enrichment of cell populations containing plasmid DNA on paramagnetic beads.

3.2.4. Enrichment of the Transfected Cell Population (Fig. 4)

1. Equilibrate the separation column carefully according to the supplier's protocol (*see Note 18*).
2. Sediment the cells at 400g at 4°C for 7 min, resuspend cells in 1 mL PBS containing 1% FCS and 2 mM EDTA.
3. Keep an aliquot of the unsorted cell fraction for reference and total cell count.
4. Place the assembled column in the magnetic separator.
5. Load the cells onto the column and wash with 3 mL PBS (*see Note 19*).
6. Collect the effluent as "nonmagnetic fraction."
7. Remove the column from the separator and flush back the retained cells.
8. Place the column in the magnetic separator and wash with 3 mL PBS containing 1% FCS and 2 mM EDTA.

9. Collect the effluent as “wash fraction.”
10. Remove the column from the separator.
11. Elute the enriched cell population.
12. Wash the cells and proceed with your protocol.

3.2.5. Genetic Immunization of Mice by Adoptive Transfer of Genetically Engineered DC

1. You should let the DC recover in the incubator for 2–3 h before carefully washing and injecting them into mice. Keep them in polypropylene tubes (slightly open) because of their tendency to adhere to plastic.
2. Place the tubes on ice to detach DC before washing them two times with PBS followed by centrifugation at 600g for 5 min with PBS. Carefully resuspend the cells each time and fill into 1 mL insulin syringes with a 30G needle.
3. We recommend to immunize mice by injecting 2.5×10^5 DC suspended in 0.5 mL of PBS via the tail vein. We are currently comparing the efficacy of intraperitoneal and subcutaneous DC injections, which would be much more simple to perform.
4. You may repeat the immunization after 7 d and monitor immune responses 7 d later.

3.3. Detection of Immune Responses

3.3.1. Detection of Antigen-Specific Antibodies

1. Obtain serum samples from mice at various time-points after gene gun immunization by tail vein bleeding. Anti- β gal antibodies can be measured easily by ELISA using r β gal as a solid-phase Ag.
2. Coat ELISA microtiter plates with 5 μ g/mL r β gal in 100 mM carbonate buffer (30 mM Na₂CO₃ and 70 mM NaHCO₃, pH 9.6) for 16 h at 4° and block with PBS containing 3% BSA and 0.05% Tween for at least 1 h.
3. Dilute serum samples serially in PBS containing 1% BSA and 0.05% Tween (assay buffer), fill into the ELISA plate and incubate 1 h at RT.
4. Detect bound antibodies using peroxidase-conjugated goat antimouse Ab (Sigma) at a 1:10,000 dilution for 1 h at RT.
5. Alternatively, you can detect γ_1 and γ_{2a} isotypes using isotype-specific goat antimouse Ab (Sigma) at a 1:10,000 dilution in assay buffer followed by peroxidase-conjugated donkey antigoat Ab (Jackson Research Lab., Bar Harbor, ME) at a 1:20,000 dilution in assay buffer.
6. Develop color using TMB (Peroxidase Substrate System, Kierkegaard and Perry, Gaithersburg, MD) according to the manufacturers instructions and read the OD450 in an ELISA plate reader (*see Note 20*).

3.3.2. Detection of Antigen-Specific Cytokine Release (ELISPOT)

1. Prepare ELISPOT plates by coating sterile Millipore HA overnight at 4°C with 10 μ g/mL purified anti-IFN- γ coating mAb in sterile PBS (50 μ L/well). Wash plates with sterile PBS and block with 200 μ L of complete medium at 37°C.

2. Harvest and pool splenocytes from two immunized mice in each group (including two nonimmunized littermates) at various time points after immunization. Mince spleens with scissors or with a stainless steel mesh, filter through 70 μm cell strainer, deplete red blood cells in lysing buffer as described for DC culture, and wash splenocytes twice in complete medium.
3. Restimulate lymphocytes in triplicates for about 22 h in ELISPOT plates at 1×10^6 and 3×10^5 cells in 200 μL of CM containing 1 $\mu\text{g/mL}$ $\beta\text{gal}_{876-884}$ peptide and 25 IU/mL recombinant human IL-2 (Chiron) per well. Include control peptides and medium alone.
4. Wash ELISPOT plates three times in PBS containing 0.1% Tween 20. Add 2.5 $\mu\text{g/mL}$ biotinylated anti-IFN- γ MAb (50 μL /well) and incubate for 2 h at 37°C.
5. Wash ELISPOT plates three times in PBS containing 0.1% Tween 20. Add SA-POD diluted 1:1000 in assay buffer (see above) for 1/2 h at RT.
6. Wash ELISPOT plates three times in PBS containing 0.1% Tween 20. Mix DAB substrate according to the manufacturer's instruction and develop color with 50 μL /well. It should take about 5 min, check under a dissecting microscope, which you can also use for quantitation.
7. Rinse the plates thoroughly under tap water and let dry overnight. You can detach the filter membranes by mounting them on an adhesive membrane normally used to seal ELISA plates.
8. Count the fuzzy spots on the filter under the dissecting microscope (see **Note 21**).

3.3.2. Detection of Antigen-Specific Cytotoxicity

1. Alternatively, you can restimulate lymphocytes in 24-well plates (at 37°C in 5% CO_2 -humidified air) at 4×10^6 cells in 2 mL of CM containing 1 $\mu\text{g/mL}$ $\beta\text{gal}_{876-884}$ peptide and 25 IU/mL recombinant human IL-2 (Chiron) per well.
2. Harvest lymphocytes after 5 d and test for their cytolytic reactivity against peptide-pulsed target cells in standard 4 h ^{51}Cr release assays using 96-well round-bottom plates. We use EL4 or C26, which grow well and are reliably loaded with chromium. You still need to be religious with the culture. The cells have to be in log phase growth and very happy. We pass them 4 and 2 d before each assay.
3. Label 2×10^6 target cells with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ for 1 h at 37°C. Wash two times. Prepare peptide-pulsed targets by addition of 1 $\mu\text{g/mL}$ peptide during radiolabeling.
4. Set up the assay in triplicate using 5000–10,000 target cells and titrated numbers of effectors in 200 μL of complete medium per well (for example 50:1, 25:1, and 12.5:1). Set up maximum release with target cells in 5% Triton-X and spontaneous release with target cells in medium only (see **Note 22**).
5. Centrifuge the plates and incubate for 4 h at 37°C and 5% CO_2 . Centrifuge again, harvest 100 μL of supernatant into titer tubes, and count in a gamma counter.
6. The percentage of ^{51}Cr release is determined by the following formula: mean experimental release-mean spontaneous release/mean maximum release-mean spontaneous release.

3.3.3. Assessment of Antitumor Immunity In Vivo: Resistance to Tumor Challenge

1. The principle goal of an effective tumor vaccine is the induction of immune protection against a tumor challenge.
2. We recommend starting tumor challenge experiments using tumor cells stably transfected with immunogenic model antigens such as β -galactosidase. You can challenge subcutaneously or intravenously (for lung metastases). Determine the minimal tumorigenic dose and the tumor growth curve. Challenge with 2–10x cells following DNA immunization. This should work reasonably well. You can then switch to your tumor antigen of choice.
3. We have successfully used genetic immunization in mice for the viral tumor antigen HPV16-E7, which is latently expressed by the HPV16-transformed murine sarcoma C3. Gene gun immunizations with a plasmid expressing HPV16-E7 or with gene gun transfected DC expressing HPV16-E7 completely protected C57BL/6 mice against a tumor challenge with C3.

4. Notes

1. A clean preparation of plasmid DNA resuspended in distilled water at 1 μ g/mL should be employed. We routinely check by restriction digest and have never had any trouble with plasmid DNA purified using Quiagen columns. CsCl-purification, however, should work equally well.
2. Depending on the hydrophobicity of the respective peptide, you should try to dissolve the peptide in a smaller amount of either PBS or DMSO. If you have problems getting the peptide into solution, you can then adjust accordingly. The DMSO content should be as low as possible (usually 10–50%), because it is toxic to cells.
3. You need to use polypropylene for DC handling. Cultured DC tend to stick to the plastic and polystyrene is even worse.
4. For a minimal FACS analysis of DC, you need to have antibodies to MHC Class II, CD11c, and CD86. The major contaminating cell types are granulocytes, B cells, and NK cells.
5. Gold particles can be obtained of different sizes and shapes. Each lot should be microscopically examined. One must bear in mind that the shape and size of the gold beads will determine their impulse and, consequently, their penetration into tissues and cells. For in vivo transfection, a mixture of different sizes may be beneficial since gold will penetrate the skin to various depths. For in vitro gene transfer to suspension cells in a monolayer, a homogenous sample of gold beads is paramount in order to reach exactly the same level of penetration.
6. This procedure sounds difficult, but is actually rather simple and foolproof. We have never had any trouble coating the gold with plasmid DNA.
7. Especially during humid weather conditions, it is important to dry the tubing first. The major problem preventing an even loading of the tubing with gold beads

appears to be residual water in the ethanol. For this reason you seal PVP and beads in 100% ethanol with parafilm and wait until they acquire room temperature before opening. Try to use fresh 100% ethanol whenever possible.

8. Loading rarely turns out perfect. The quality of the DNA appears to influence the fine distribution of the beads in the cartridge. Surprisingly, even rather unevenly loaded cartridges work well for immunization. You can test the DNA precipitation onto the gold beads. Place three bullets in a 1.5 mL microfuge with 600 μ L distilled water. Vortex, sonicate, rinse, and centrifuge. Measure OD₂₆₀. It should be close to 0.05 (= 1.5 μ g/mL).
9. You can also test the cartridges by discharge onto parafilm or into agar plates (you can use plates to grow bacteria or prepare some with 3% water agar). Cut sections and observe the distribution of gold beads under the microscope.
10. We have had best results at 400 psi in mice. You should start the experiments with β -gal and EGFP as marker genes and simultaneous test antigens. Expression in the skin can be conveniently detected and immune responses analyzed.
11. Immunodepletion of contaminant cells can be performed on the bone marrow cell suspension using hybridoma supernatants (0.5–1 mL of anti-B220, ATCC TIB 146, anti-IA, anti GR-1) and either magnetic beads or rabbit complement according to standard protocols. In our hands, this has not consistently increased the purity of the DC culture. However, it can reduce the yield considerably, depending on the reagents.
12. DC can be enriched by density gradient centrifugation over metrizamide gradients (14.5 mg/mL complete medium). There is considerable debate how to obtain the best and most immunostimulatory DC population. The addition of CD40L or TNF- α during the last 2 d of culture has been reported to increase the percentage of dendritic cells expressing high levels MHC Class II and B7.2. These cultures have been more efficient *in vivo*.
13. For phenotyping, DC are washed in PBS supplemented with 2%FCS, 1 mM EDTA, 0.1% NaN₃ (FACS-staining buffer) and incubated (30 min at 4°C) with one of the following mAb: Isotype controls, anti-IA^b or -IA^d, anti-CD40, anti-B7.1 (CD80), anti-B7.2 (CD86), and anti-CD11c for the detection of dendritic cells. Contaminating cells can be visualized by using anti-B220, anti-CD3, anti-GR-1, and anti-NK1.1 (C57BL/6 only). Antibodies are either directly PE-/FITC-conjugated or a secondary antibody is used.
14. We have used the prototype Accell helium pulse gun kindly provided by Geniva (Middleton, WI). We have not extensively tested the Helios Gene Gun available from Bio-Rad for this purpose yet. Overall, transgene expression has been rather low when bombarding primary dendritic cell cultures as assessed by luciferase assays. However, gene gun transduction has been very reliable in our hands when compared to other nonviral gene delivery methods. We recommend to use one of the longterm dendritic cell lines which have been established in various labs (18).
15. To avoid clumping of the gold particles, wash them two times in 70% ethanol p.a and two times in aqua ad in inectabilia under sterile conditions.

16. Plasmid DNA needs to be prepared under sterile conditions. We routinely use endotoxin free plasmid preps (Qiagen, Chatsworth, CA) and dilute plasmids in aqua ad iniectabilia under sterile conditions.
17. When loading the macrocarrier with gold particles, do not let the gold dry out too much.
18. Avoid air bubbles in the column, when equilibrating the column. Do not load aggregated cells onto the column; resuspend or filter cells carefully before loading.
19. If buffer or cell suspension does not flow well tap hard onto the column to liberate air bubbles or cell clumps in the column, capillary or flow resistor. Use capillary G23 as flow resistor.
20. Any number of detection reagents can be used. Secondary reagents have to be titrated to yield optimal results and low background. In BALB/c mice, the relative amount of antibodies of the IgG1 and IgG2a isotype is indicative of a Th2 versus a Th1-type immune response, respectively. Care must be taken to exclude cross-reactivity of isotype-specific secondary antibodies.
21. We have adopted our ELISPOT from Schneider, et al. (40). When assaying splenocytes from DC-immunized mice, you need to enrich for CD8⁺ T cells, because there is considerable background IFN γ release in whole splenocyte suspensions, presumably resulting from the FCS which is normally used for DC culture. We are currently developing protocols to replace FCS with NMS in DC culture and/or ELISPOT.
22. Anti-CD4 and anti-CD8 MAB (hybridoma supernatants or purified antibody) followed by separation with magnetic beads or complement mediated depletion can be used to remove (or enrich) effectors.

References

1. Tang, D. C., DeVit, M. J., and Johnston, S. A. (1992) Genetic immunization: a simple method for eliciting an immune response. *Nature* **356**, 152–154.
2. Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. (1997) DNA vaccines. *Ann. Rev. Immunol.* **15**, 617–648.
3. Tüting, T., Storkus, W. J., Faló, L. D. (1998) DNA immunization targeting the skin: Molecular control of adaptive immunity. *J. Invest. Dermatol.* **111**, 183–188.
4. Corr, M., Lee, D. J., Carson, D. A., and Tighe, H. (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* **184**, 1555–1560.
5. Fu, T-M., Ulmer, J. B., Caulfield, M. J., Deck, R. R., Friedman, A., Wang, S., et al. (1997) Priming of cytotoxic T lymphocytes by DNA vaccines: requirements for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Med.* **3**, 362–371.
6. Iwasaki, A., Torres, C. A. T., Ohashi, P. S., Robinson, H. L., and Barber, B. H. (1997) The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* **159**, 11–14.
7. Doe, B., Selby, M., Barnett, S., Baenziger, J., and Walker, C. M. (1996) Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA

- is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* **93**, 8578–8583.
8. Cella, M., Sallusto, F., and Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* **9**, 10–16.
 9. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245–252.
 10. Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K., and Falo, L. D. (1996) DNA-based immunization by in vivo transfection of dendritic cells. *Nature Med.* **2**, 1122–1128.
 11. Porgador, A., Irvine, K. R., Iwasaki, A., Barber, B. H., Restifo, N. P., and Germain, R. N. (1998) Predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after gene gun immunization. *J. Exp. Med.* **188**, 1075–1082.
 12. Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. (1999) DNA vaccination: Transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med.* **189**, 169–177.
 13. Casares, S., Inaba, K., Brumeanu, T-D., Steinman, R. M., and Bona, C. A. (1977) Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* **186**, 1481–1486.
 14. Walker, P. S., Scharton-Kersten, T., Rowton, E. D., Hengge, U., Boulloc, A., Udey, M. C., and Vogel, J. C. (1998) Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type immune response and protection in a murine model of leishmaniasis. *Hum. Gene. Ther.* **9**, 1899–1907.
 15. Labeur, M. S., Roters, B., Pers, B., Mehling, A., Luger, T. A., Schwarz, T., and Grabbe, S. (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* **162**, 168–175.
 16. Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J., and Enk, A. H. (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* **27**, 3135–3142.
 17. Tüting, T., DeLeo, A. B., Lotze, M. T., and Storkus, W. J. (1997) Bone marrow-derived dendritic cells genetically modified to express tumor-associated antigens induce antitumor immunity in vivo. *Eur. J. Immunol.* **27**, 2702–2707.
 18. Timares, L., Takashima, A., and Johnston, S. A. (1998) Quantitative analysis of the immunopotency of genetically transfected dendritic cells. *Proc. Natl. Acad. Sci. USA* **95**, 13,147–13,152.
 19. Tüting, T., Wilson, C. C., Martin, D., Kasamon, Y., Rowles, J., Ma, D. I., et al. (1998) Autologous human monocyte-derived dendritic cells genetically modified to express melanoma antigens elicit primary cytotoxic T cell responses in vitro: Enhancement by cotransfection of genes encoding the Th1-biasing cytokines IL-12 and IFN- α . *J. Immunol.* **160**, 1139–1147.

20. Conry, R. M., Widera, G., LoBuglio, A. F., Fuller, J. T., Moore, S. T., Barlow, D. L., et al. (1996) Selected strategies to augment polynucleotide immunization. *Gene Ther.* **3**, 67–74.
21. Irvine, K. R., Rao, R. B., Rosenberg, S. A., and Restifo, N. P. (1996) Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. *J. Immunol.* **156**, 238–245.
22. Tüting, T., Gambotto, A., Storkus, W. J., De Leo, A. B. (1999) Co-delivery of T helper 1-biasing cytokine genes enhances the efficacy of gene gun immunization of mice: Studies with the model tumor antigen β -galactosidase and the BALB/c Meth A p53 tumor-specific antigen. *Gene Ther.* **6**, 629–636.
23. Corr, M., Tighe, H., Lee, D., Dudler, J., Trieu, M., Brinson, D. C., and Carson, D. A. (1997) Costimulation provided by DNA Immunization enhances antitumor immunity. *J. Immunol.* **159**, 4999–5004.
24. Gurunathan, S., Irvine, K. R., Wu, C-Y., Cohen, J. I., Thomas, E., Prussin, C., et al. (1998) CD40ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol.* **161**, 4563–4571.
25. Dyall, R., Bowne, W. B., Weber, L. W., LeMaout, J., Szabo, P., Moroi, Y., et al. (1998) Heteroclitic immunization induces tumor immunity. *J. Exp. Med.* **15**, 553–1561.
26. Parra-Lopez, C. A., Lindner, R., Vidavsky, I., Gross, M., and Unanue, E. R. (1997) Presentation on class II MHC molecules of endogenous lysozyme targeted to the endocytic pathway. *J. Immunol.* **158**, 2670–2679.
27. Boon, T. and Van der Bruggen, P. (1996) Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* **183**, 725–729.
28. Rosenberg, S. A. (1997) Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol. Today* **18**, 175–182.
29. Syrengelas, A. D., Chen, T. T., and Levy, R. (1996) DNA immunization induces protective immunity against B-cell lymphoma. *Nat. Med.* **2**, 1038–1041.
30. Rosato, A., Zambon, A., Milan, G., Ciminale, V., D'Agostino, D. M., Macino, B., et al. (1997) CTL response and protection against P815 tumor challenge in mice immunized with DNA expressing the tumor-specific antigen P815. *Hum. Gene Ther.* **8**, 1451–1458.
31. Schreurs, M. W., de Boer, A. J., Figdor, C. G., and Adema, G. J. (1998) Genetic vaccination against the melanocyte lineage-specific antigen gp100 induces cytotoxic T lymphocyte-mediated tumor protection. *Cancer Res.* **58**, 2509–2514.
32. Weber, L. W., Bowne, W. B., Wolchok, J. D., Srinivasan, R., Qin, J., Moroi, Y., et al. (1998) Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J. Clin. Invest.* **102**, 1258–1264.
33. Tüting, T., Gambotto, A., De Leo, A. B., Robbins, P. D., Lotze, M. T., and Storkus, W. J. (1999) Induction of tumor antigen-specific immunity using DNA immunization in mice. *Cancer Gene Ther.* **6**, 73–80.

34. Ugen, K. E., Nyland, S. B., Boyer, J. D., Vidal, C., Lera, L., Rasheid, S., et al. (1998) DNA vaccination with HIV-1 expressing constructs elicits immune responses in humans. *Vaccine* **16**, 1818–1821.
35. MacGregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., et al. (1998) First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J. Infect. Dis.* **178**, 92–100.
36. Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A. C., Sandstrom, E., and Wahren, B. (1998) Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* **351**, 1320–1325.
37. Wang, R., Doolan, D. L., Pe, T. P., Hedstrom, R. C., Coonan, K. M., Charoenvit, Y., et al. (1998) Induction of antigen-specific cytotoxic T lymphocytes by a malaria DNA vaccine. *Science* **282**, 476–480.
38. Feltkamp, M. C. W., Smis, H. L., Vierboom, M. P. M., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., et al. (1993) Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur. J. Immunol.* **23**, 2242.
39. Brattain, M. G. et al. (1980) Establishment of mouse colonic carcinoma cell lines with different metastatic properties. *Cancer Res.* **40**, 2142–2146.
40. Schneider, J., Gilbert, S., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., et al. (1998) Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Med.* **4**, 397–402.



<http://www.springer.com/978-0-89603-714-4>

Gene Therapy of Cancer
Methods and Protocols
Walther, W.; Stein, U. (Eds.)
2000, XVI, 645 p., Hardcover
ISBN: 978-0-89603-714-4
A product of Humana Press