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Generation of High Specificity Antibodies against Membrane Proteins Using DNA-Gold Micronanoplexes for Gene Gun Immunization

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Abstract

Membrane proteins are the molecular interface of the cell and its environs, however studies are highly technically challenging, mainly due to stability of the isolated protein. Towards the production of antibodies that recognize properly-folded and stabilized forms of membrane protein antigen, we describe a DNA-based immunization method for mice that expresses the antigen in the membranes of dendritic cells, thus allowing direct presentation to the immune system. This genetic immunization approach employs a highly efficient method of biolistic delivery based on DNA-gold micronanoplexes, which are complexes of micron-sized gold particles that allow

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KEY REFERENCE

Chambers and Johnston, 2003. See above.

First description of the efficiency of genetic immunization towards the production of polyclonal antibodies against soluble proteins.

Hansen et al., 2016. See above.

First description of the efficiency of genetic immunization towards the production of polyclonal antibodies against membrane proteins.

Liu et al., 2016. See above.

Recent review of DNA-based immunization methods specific for antibody production.

Svarovsky et al., 2009.

See above. *First description of DNA-gold micronanoplexes.*

INTERNET RESOURCES (optional)

dermal penetration and nanometer-sized gold particles that provide a higher surface area for DNA binding than micron gold alone. In contrast to antibodies derived from immunizations with detergent-solubilized protein or with protein fragments, antibodies from genetic immunization are expected to have a high capacity for binding conformational epitopes and for modulating membrane protein activity.

Keywords

antibodies; membrane protein; genetic immunization; DNA immunization; conformational epitopes; gene gun

INTRODUCTION

The intense interest in membrane proteins (The International Transporter Consortium et al., 2010; Bagal et al., 2013; Andrews et al., 2014) is borne out by the fact that these macromolecules are the targets of an estimated 60% of approved drugs (Yildirim et al., 2007). Membrane protein targets include G protein-coupled receptors (GPCRs), ion channels and transporters. Because many membrane protein targets consist of highly related proteins with different functions, there is a particular interest in employing the potential exquisite specificity of antibodies for the development of therapeutics. Target-specific antibodies have also enabled the structure determination of more than two dozen unique membrane protein structures by co-crystallization (Lieberman et al., 2011; Bukowska and Grütter, 2013) and by cryo-electron microscopy (Wu et al., 2012; Lee et al., 2016).

Membrane proteins present unique difficulties for antibody production given the constrained three-dimensional presentation and limited solvent exposure of the available epitopes (Satofuka et al., 2014). Antibodies derived from peptide-based immunizations have proven unsuitable for membrane protein co-crystallization due to the inability to recognize these conformational epitopes (Hamakubo et al., 2014). Detergents do not adequately stabilize membrane proteins during immunization and can also mask critical epitopes (Wilkinson et al., 2015). In addition, many membrane proteins require unique lipids for stability and activity (Lee, 2004; Zhou and Cross, 2013; Poveda et al., 2014). The isolation of functional antibodies (i.e., antibodies that modulate protein function) is particularly dependent on the availability of properly-folded and stabilized membrane protein antigen (Wilkinson, 2016). Here, we present an antibody production method that is based on membrane protein antigen that is expressed *in vivo* and is membrane-embedded.

Genetic immunization, also called DNA immunization, is an obvious route to antibody production for membrane proteins because the gene-immunized host expresses, folds, and modifies the membrane protein antigen within a native membrane environment. Thus, genetic immunization is expected to allow immune recognition of the myriad in-membrane interactions with other macromolecules as well as of intermediate assembly states of the membrane protein. Because many membrane proteins are difficult to purify and stabilize, genetic immunization also precludes the need to maintain stable purified antigen in sufficient quantity and quality over the course of an immunization schedule.

Genetic immunization produces an immune response from DNA that encodes the antigen. This method has applications in both antibody production [reviewed in (Liu et al., 2016)] and vaccine development [reviewed in (Li et al., 2012)]. DNA-based immunization methods include simple needle injection of naked DNA, biolistic delivery of DNA-coated particles, transdermal patches, and electroporation-based methods. This protocol uses biolistic delivery, although we note that the same DNA vectors may be applicable to any DNA-based method (Williams, 2014). Biolistic methods deliver nucleic acids intracellularly following precipitation of circular or linear DNA constructs onto micron-sized gold particles. The DNA-gold is adhered to the inner surface of short pieces of Tefzel tubing, forming the “bullets” that are used in a commercially-available gene gun. Upon delivery, the DNA-gold is propelled through the bullet *via* a high-pressure burst of helium gas. The DNA-gold particles penetrate the epidermal cells, which include keratinocytes and, importantly, dendritic cells (Condon et al., 1996), the antigen-presenting cells of the immune system. Upon exposure to antigen, dendritic cells migrate to the lymph nodes to elicit an adaptive immune response. Biolistic immunization therefore leads to direct DNA transfection of dendritic cells (Condon et al., 1996) and *in vivo* expression of the protein in both the dermal tissues and the lymph nodes (Williams et al., 1991; Condon et al., 1996).

This unit describes a DNA-based immunization method that is expected to generate conformation-dependent antibodies against membrane-embedded proteins. The membrane protein is not required for the immunization process, only for characterizing the resulting antibodies. The first protocol details the generation of bullets that consist of DNA-gold micronanoplexes (Basic Protocol 1) and the second protocol describes a typical immunization scheme (Basic Protocol 2). Micronanoplexes are complexes of micron-sized gold particles, which allow dermal penetration, and nanometer-sized gold particles, which provide a higher surface area for DNA binding than micron-sized gold alone. The micronanoplex approach should be applicable to other animal species with appropriate expression vectors (Liu et al., 2016). The protocols in this unit can be accompanied by standard methods for serum collection, terminal bleeds, isolation of B cells, and production of monoclonal antibodies. The resulting conformational antibodies are expected to allow achievement of unique atomic-level membrane protein structures and subsequent development of structure-based and antibody-based therapeutics.

STRATEGIC PLANNING

Plasmid DNAs

Plasmid DNAs for this process include (1) an immunization vector that is designed to optimally express and encode the target membrane protein and (2) vectors that express and encode adjuvants (Table 1). The immunization vectors are non-replicating and non-selectable in the mouse. Vector propagation is achieved in *Escherichia coli* due to a bacterial origin and a selectable marker. Immunization vectors that are specific for membrane proteins are still under development (see Commentary). By comparison, for proteins lacking transmembrane domains, there is much more information on the optimal vector components and fusion sequences [reviewed in (Liu et al., 2016)]. This distinction is made because membrane protein assembly requires the additional steps of membrane targeting and

membrane integration. Membrane-targeting sequences are generally located at the N-terminus and may also be necessary for the structural integrity of the membrane protein, thus the effect of such fusions may be protein-dependent. Still under evaluation for membrane protein antigens is the effect of additional genetically-encoded fusion sequences.

We recently reported the first description of the efficiency of any DNA immunization approach specific for membrane proteins (Hansen et al., 2016). We applied the DNA-gold micronanoplex method (Svarovsky et al., 2009) and the immunization vector pCMVi-LSrCOMPTT (Table 1; Figure 1) to a structurally diverse set of 17 membrane proteins from two Biosafety Level 3 pathogens, and demonstrated polyclonal antibody production for more than half of the targets (Hansen et al., 2016). A major validation of this study was that antibody production correlated with prior reports of target immunogenicity in infected or immunized hosts (Hansen et al., 2016). To support widespread use, all plasmid vectors from this study have been made available through the non-profit DNASU Plasmid Repository (Seiler et al., 2014).

In the pCMVi-LSrCOMPTT immunization vector (Figure 1), antigen expression is driven by the cytomegalovirus (CMV) immediate-early promoter with intron (Chambers and Johnston, 2003) and a strong Kozak sequence (Hansen et al., 2016). The target membrane protein is expressed with the following N-terminal fusion: a 24 amino acid membrane-targeting leader sequence (LS) from the human α_1 -antitrypsin gene (Long et al., 1984); a 23 amino acid randomly-generated (r) immunogenic peptide (Chambers and Johnston, 2003); a 45 amino acid cartilage oligomeric matrix protein (COMP) assembly domain from the pentameric rat matrix protein (Terskikh et al., 1997); and a 50 amino acid sequence containing tetanus toxoid epitopes (TT) for overcoming humoral tolerance (King et al., 1998; Chambers and Johnston, 2003). Vector pCMVi-LS (Table 1) allows fusion to only the LS leader sequence (Sykes and Johnston, 1999). Transcription is terminated by the human growth hormone terminator sequence. The target gene can be cloned in frame using nine unique restriction sites (Figure 1). It is expected that optimized gene sequences may improve antibody titers. These vectors can also be used for non-transmembrane proteins because the leader sequence targets the antigen for secretion (Chambers and Johnston, 2003).

Adjuvant vectors, i.e., vectors that encode proteins with known adjuvant activity, may increase antibody titers. Our recent report (Hansen et al., 2016) included pCMVi-LS-LTA-R192G and pCMVi-LS-LTB (Table 1), which encode mouse-codon-optimized sequences of the enterotoxigenic *E. coli* heat-labile enterotoxin subunits A (inactive mutant R192G) and B that are fused to the α_1 -antitrypsin leader sequence (Whitlock et al., 2011). These vectors have also been applied to vaccine development (Whitlock et al., 2011); however, the effect of these vectors on antibody titers has not been specifically evaluated (Whitlock et al., 2011; Hansen et al., 2016). For soluble (i.e., non-transmembrane) proteins, inclusion of the adjuvant vectors pCMVi-mGMCSF and pCMVi-Flt3L (Table 1) led to improved titers (Chambers and Johnston, 2003). Additional adjuvant vectors that have been applied to vaccine studies were also shown to increase antibody responses (Scheerlinck, 2001; Sasaki et al., 2003; Kobiyama et al., 2013).

Plasmid DNAs can be purified using QIAprep Spin Miniprep Kits (Qiagen #27106) or Plasmid Maxi Kits (Qiagen #12162). DNAs can be suspended in molecular grade water or in 10 mM Tris-Cl, pH 8.5, and stored at -20°C . The effect of endotoxin-free preparations of DNA has not been evaluated for this protocol.

CpG oligodeoxynucleotide adjuvants

Unmethylated CpG oligodeoxynucleotides (ODNs) stimulate a humoral responses by mimicking the presence of foreign DNA [reviewed in (Bode et al., 2011)]. CpGs can be included with plasmid DNA during incorporation into bullets (Hansen et al., 2016). CpG oligodeoxynucleotides with complete phosphorothioate linkages, which resist nuclease digestion, can be purchased (e.g., Invitrogen or Sigma) in desalted form and without further purification. The sequence for Class B CpG “ODN 2006” is 5′–TCGTCGTTTTGTCGTTTTGTCGTT–3′ (Hartmann et al., 2000) and for Class C CpG “ODN 2395” is 5′–TCGTCGTTTTCGGCGCGCCG–3′ (Vollmer et al., 2004). Suspend CpGs in molecular grade water and store at -20°C .

BASIC PROTOCOL 1 PREPARATION OF DNA-GOLD MICRONANOPLEX BULLETS FOR GENE GUN IMMUNIZATION

This protocol describes preparation of DNA-gold micronanoplex bullets (Svarovsky et al., 2009; Hansen et al., 2016) for the Bio-Rad Helios gene gun. Bullets for the gene gun consist of short pieces of flexible (Tefzel) tubing that are coated on the inner surface with DNA-bound gold particles. The gene gun uses a burst of helium gas to propel the DNA-gold particles into the cells of the dermal tissues, thus allowing *in vivo* expression of the encoded protein. Historically, these bullets have been prepared by calcium/spermidine precipitation of DNA onto micron-sized gold particles. The DNA-gold micronanoplex method differs primarily by substituting polyethylenimine for calcium/spermidine (Svarovsky et al., 2008) and by the addition of nanometer-sized gold particles, which allows a greater than ten-fold increase in DNA binding versus micron-sized gold alone (Svarovsky et al., 2009). These DNA-gold micronanoplexes have been applied to the identification of vaccine antigens (Whitlock et al., 2011; Shen, 2012), to the development of electrochemical immunosensors for biomarkers (Mars et al., 2016), and to antibody production for membrane proteins (Hansen et al., 2016). We expect that these high-load bullets can also be applied to antibody production for non-transmembrane antigens. To achieve DNA-gold micronanoplex production, micron-sized (1–2 μm) gold particles are thiol-functionalized (Figure 2A) and then activated for amide bond linkage to polyethylenimine to form microgold-PEI (Figure 2B). In a separate reaction, molecular gold (HAuCl_4) is modified with cysteamine to form nanogold (Figure 2C), and the nanogold is combined with DNA to form DNA-nanogold (Figure 2D). The microgold-PEI and DNA-nanogold are then mixed to form DNA-gold micronanoplexes (Figure 2E), which are adhered to the tubing to form the bullets for the gene gun.

Materials

Reagents

Tefzel tubing, OD 0.125 in., ID 0.093 in., 50 ft. per coil; Bio-Rad #165-2441 or K-mac Plastics #KM-5374)

Ethanol, 200 proof

Acetone

Helium compressed gas, 99.99%, with helium pressure regulator (Bio-Rad #165-2413) that is specific for use with the Helios gene gun

Silicone (adapter) tubing, OD 5/32 in., ID 3/32 in., wall 1/32 in., 50 ft. per coil (e.g., Tygon 3350, Saint-Gobain #ABW00004); this tubing fits over the ends of the Tefzel tubing

Gold particles, unmodified, 1–2 μm diameter (e.g., Bio-Rad #165-2264)

250 mL glass Erlenmeyer flask

Concentrated sulfuric acid (98%)

30% hydrogen peroxide, ACS grade

50 mL disposable conical polypropylene tubes suitable for centrifugation at 900 $\times g$

11-mercaptoundecanoic acid, 95% (MUDA; e.g., Sigma-Aldrich #450561; top off opened bottle with nitrogen gas, wrap lid with Parafilm, and store under desiccant [see Troubleshooting])

Nitrogen compressed gas, with nitrogen gas regulator

Parafilm M

Desiccant (e.g., Drierite 10–20 mesh, indicating)

Containers for storage of chemicals under desiccant at room temperature, at 4°C, and at –20°C

Aluminum foil

0.1 M MES, pH 6.0 (see recipe)

Filters (0.22 μm) for sterilization of 0.1 M MES, pH 6.0 (e.g., Millipore bottle top filter, polyethersulfone, #SCGPT01RE)

N-hydroxysuccinimide (NHS), 98% (e.g., Sigma #130672; top off opened bottle with nitrogen gas, wrap lid with Parafilm, and store under desiccant [see Troubleshooting])

N-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC; e.g., Sigma #E1769; top off opened bottle with nitrogen gas, wrap lid with Parafilm, store at –20°C under desiccant, and use within 6 months of purchase [see Troubleshooting])

10% polyethylenimine (PEI), pH 9 (see recipe)

Dry ice (or –80°C freezer)

Kimwipes

508 mM H₂AuCl₄ (see recipe)

Micro magnetic stir bar, PTFE coated, 3 mm diameter, 10 mm length (e.g., Fisher #14-513-65SIX)

Cysteamine, 95% (CAS number 60-23-1; e.g., Sigma #30070, or Santa Cruz #sc-217991); store at 4°C under desiccant; use a recently-ordered, unopened bottle

Sodium borohydride, 99.99% (NaBH₄; e.g., Sigma 480886; store under desiccant)

Single-edge razor blades

10 mL syringes; non-Luer Lok is recommended

An ethanol-resistant marker is recommended for labeling samples for steps during and after the preparation of DNA-nanogold

Purified plasmid DNAs (see Strategic Planning)

CpG oligodeoxynucleotides (see Strategic Planning and Troubleshooting)

Molecular grade water

15 mL and/or 1.5 mL sterile disposable conical polypropylene centrifugation tubes (15 mL tubes that have snap-caps, e.g., Nunc #362694, will facilitate later steps during micronanoplex and bullet production)

1-butanol, ACS grade

Forceps for handling bullets

Equipment

Vacuum line connected to two sequential vacuum trap flasks

Chemical fume hood

Flowmeter for helium gas, capable of 5 L/min flowrate (e.g., Cole-Parmer #EW-68561-57)

Analytical balance

Tube vortexer

Centrifuge for 50 mL polypropylene tubes, capable of $900 \times g$

Microtube mixer capable of 1400 rpm (e.g., Eppendorf Thermomixer)

Lyophilizer

–80°C freezer (or dry ice)

Centrifuge for 15 mL and 1.5 mL polypropylene tubes, capable of $100 \times g$

Magnetic stir plate

Helios gene gun system Tubing Cutter (Bio-Rad #165-2422) with single-edge razor blades; the Tubing Cutter is used to prepare the 1 cm length bullets

Prepare Tefzel tubing

Unless noted, all protocol steps are performed at room temperature and use deionized, distilled water.

- 1 Wash the Tefzel tubing with, per tubing coil, 200 mL of ethanol by pulling the solution through using a vacuum connected to two sequential vacuum trap flasks.

The first trap collects the solution. The second trap protects the vacuum source.

Each lot of Tefzel tubing should be tested for its suitability for bullet-production.

Multiple coils of Tefzel tubing can be run simultaneously by connecting the coils in series using silicone adapter tubing.

CAUTION: Ethanol is flammable. Follow proper guidelines during use and disposal.

- 2 Wash the Tefzel tubing with, per tubing coil, 200 mL of 100% acetone.

CAUTION: Acetone is highly flammable. Follow proper guidelines during use and disposal.

- 3 In a chemical fume hood, dry the Tefzel tubing for 20 minutes under a flow of helium gas at 5 L/min and with the helium pressure regulator set at 20 psi. Do not continue to dry beyond 20 minutes. Visually inspect that the Tefzel tubing lacks liquid droplets. If liquid persists, repeat the acetone rinse.

Dry each coil independently; do not connect the coils in series.

CAUTION: Transport, storage and set-up of compressed gas systems should be handled only by trained personnel.

- 4 Store the Tefzel tubing in enclosed circles by connecting the open ends of each coil with a piece of silicone adapter tubing.

Prepare microgold-PEI

Micron-sized gold particles (microgold-PEI) will be cleaned with piranha solution (Figure 2A) and then coated sequentially with 11-mercaptoundecanoic acid (MUDA; Figure 2A) and polyethylenimine (PEI; Figure 2B). Due to gradual loss of DNA binding capacity, microgold-PEI should be used within 3 months. Per bullet, 1 mg of microgold-PEI will be needed.

- 5 At least one day before bullet production, add 2 g of unmodified micron gold particles to a clean 250 mL glass Erlenmeyer flask.

Perform the next 5 steps in a chemical fume hood. The micron gold will be cleaned with piranha solution (sulfuric acid and hydrogen peroxide; Figure 2A).

CAUTION: Piranha solution is highly corrosive and extremely oxidizing.

- 6 Add 15 mL of concentrated sulfuric acid to the flask.

Sulfuric acid is extremely caustic. Use proper safety precautions and waste disposal.

- 7** While gently mixing the solution using orbital rotation by hand, slowly add in a dropwise manner a total of 5 mL of 30% hydrogen peroxide.

Gradual addition of hydrogen peroxide is necessary to avoid an out-of-control reaction due to exothermic heating and evolution of gas.

Tilt the flask slightly so that the flask opening points away from oneself.

CAUTION: Hydrogen peroxide is a strong oxidizer and is corrosive. Use proper safety precautions and waste disposal.

- 8** Allow the solution to cool to room temperature without further mixing, approximately 20 min.

- 9** Discard the supernatant by decanting.

Wrap the flask in paper towels to catch any drips upon decanting.

CAUTION: Do not store piranha solution waste in glass containers or in enclosed containers. Before using piranha solution, check local waste storage requirements.

- 10** Wash the gold by brief orbital mixing with 50 mL of water. When the gold settles, discard the supernatant by decanting. Repeat twice; before decanting all of the final supernatant, transfer the gold solution to a 50 mL polypropylene tube and then discard the remaining supernatant by decanting.

- 11** Wash the gold with 40 mL of water and mixing by vortex. Discard the supernatant by decanting. Repeat four times.

- 12** Wash the gold with 20 mL of ethanol and mixing by vortex. The gold will settle less quickly at this step. To facilitate settling, centrifuge at 90

g for 2 minutes. Discard the supernatant by pipetting. Repeat once.

- 13** Add 20 mL of ethanol to the gold and mix by vortex.

- 14** Add 0.5 g of MUDA and mix by vortex.

MUDA allows alkanethiol modification of the micron gold surface (Figure 2A).

- 15** Wrap the tube lid in Parafilm to minimize leakage. Wrap the tube in foil to protect the solution from exposure to light.

- 16** Attach the tube horizontally to the platform of a microtube mixer and shake the solution at 1400 rpm for 2 h.

Use tape to secure the tube horizontally on top of the microtube mixer.

During this time, bring EDC to room temperature.

- 17** Centrifuge the gold solution at $900 \times g$ for 2 min.

- 18** Discard the clear supernatant by pipetting.

- 19** Add 40 mL of water to the gold, mix by vortex, centrifuge at $900 \times g$ for 2 min, and discard the supernatant by decanting. Repeat once.

Some gold powder may adhere to the tube walls, but the supernatant should be clear following centrifugation.

- 20** Add 20 mL of 0.1 M MES, pH 6.0, to the gold and suspend by vortex.

- 21** Add 300 mg of NHS to the gold solution and mix by vortex.

- 22** Add 200 mg of EDC to the gold solution and mix by vortex.

NHS and EDC activate the carboxylic acid groups of MUDA (Figure 2B).

CAUTION: EDC can cause serious eye damage. Wear the proper personal protective equipment.

- 23** Wrap the tube lid in Parafilm and wrap the tube in foil. Attach the tube horizontally to the platform of a microtube mixer and shake the solution at 1400 rpm for 30 min.

During this time, prepare a fresh 20 mL solution of 10% PEI, pH 9 (see recipe).

- 24** Centrifuge the gold slurry at $900 \times g$ for 2 min and discard the supernatant by pipetting.

- 25** Add the 20 mL solution of 10% PEI, pH 9, to the gold pellet and vortex briefly to mix.

- 26** Wrap the tube lid in Parafilm, wrap the tube in foil, and shake the solution horizontally at 1400 rpm for 2 h.

During this time, turn on the lyophilizer to achieve a vacuum.

- 27** Centrifuge the microgold-PEI solution at $900 \times g$ for 2 min and discard the supernatant using a pipet.

At this point, the gold suspends easily, so supernatants must be removed by pipetting, not decanting.

- 28** Wash the microgold-PEI with 40 mL of water by mixing by vortex, centrifuging at $900 \times g$ for 2 min, and discarding the supernatant using a pipet. Repeat once.

- 29** Freeze the microgold-PEI at -80°C for 30 min. Alternatively, freeze the sample in an ethanol/dry ice bath while rotating the tube slowly for approximately 2 min.

CAUTION: Dry ice and dry ice baths can cause cryogenic burns. Dry ice releases large volumes of carbon dioxide gas as it sublimates.

- 30** For lyophilization, remove the tube cap. To avoid loss of the sample, cover the open tube end appropriately, such as with a Kimwipe secured with a rubber band. Lyophilize the microgold-PEI overnight and in the dark.

Upon complete drying, the microgold-PEI should look like a dry powder.

Apply and remove the vacuum slowly to avoid loss of the powdered microgold-PEI.

- 31** Before storage, carefully top off the tube airspace with nitrogen. Seal the tube with Parafilm and cover the tube with foil to prevent light exposure. Store at 4 °C under desiccant. Use within 3 months due to gradual loss of binding capacity.

The yield of microgold-PEI (by weight) is approximately 75% of the amount of micron gold weighed at step 5.

Prepare nanogold

Nanogold will be prepared by functionalization of molecular gold (HAuCl₄) with cysteamine (Figure 2C).

- 32** On the day of bullet preparation, prepare a 1.59 mM solution of HAuCl₄ by adding 62.5 µL of the 508 mM HAuCl₄ solution to 20 mL of molecular grade water in a 50 mL polypropylene tube that contains a micro magnetic stir bar. Wrap the tube in foil to protect the solution from light. Mix the solution using a magnetic stir plate.

The 1.59 mM HAuCl₄ solution will be bright yellow.

- 33** Prepare at least 200 µL of a fresh solution of 213 mM cysteamine (16.4 mg/mL). Due to rapid oxidation of cysteamine, the cysteamine solution should be freshly prepared from a recently-ordered, unopened bottle that has been maintained under desiccant.

- 34** Upon vigorous stirring of the 1.59 mM HAuCl₄ solution, slowly add dropwise 200 µL of 213 mM cysteamine. Replace the lid to protect the solution from light.

The yellow solution will turn a dark yellow/brown, and then return to yellow.

- 35** In a 50 mL polypropylene tube, prepare a fresh solution of 10 mM NaBH₄ (0.372 mg/mL). Immediately loosen the cap on this solution after mixing due to build-up of hydrogen gas.

CAUTION: NaBH₄ causes severe skin burns and eye damage. In contact with water, NaBH₄ releases flammable gases which may ignite spontaneously. In case of fire, use dry sand, dry chemical, or alcohol-resistant foam for extinction, not water.

NaBH₄ acts here as a reducing agent (Figure 2C).

- 36** After 10 min of continuous stirring of the HAuCl₄/cysteamine solution, rapidly inject 5 µL of 10 mM NaBH₄ into the stirring solution.

The solution will turn black where NaBH₄ has been added and then will quickly return to yellow.

- 37** Continue mixing the nanogold suspension (1.59 mM HAuCl₄, 2.13 mM cysteamine, 2.5 µM NaBH₄) for 30–45 min.

The solution will remain yellow for approximately 5 min and will eventually turn a deep wine red, indicating the formation of gold particles of ~36 nm size [Figure 2C; (Niidome et al., 2004; Svarovsky et al., 2009)].

At the end of 45 min, the deep wine red solution should appear clear. Do not use a cloudy solution for the remaining steps. A cloudy solution indicates poor quality H_{Au}Cl₄ or cysteamine.

- 38** Top off the nanogold suspension (1.59 mM H_{Au}Cl₄, 2.13 mM cysteamine, 2.5 μM NaBH₄) with nitrogen gas, wrap the lid in Parafilm, and store the solution at 4°C and in the dark. Use within 48 h.

Prepare individual lengths of Tefzel tubing

This protocol uses 10 mL syringes to draw the DNA-nanogold suspension into the Tefzel tubing.

- 39** Use a single-edge razor blade to cut the washed and dried Tefzel tubing to lengths of 25–33 cm.

This tubing length allows (i) later circularization of the tubing, (ii) uniform adherence of the DNA-nanogold, and (iii) effective drying of the DNA-nanogold under helium gas flow.

If the end of the tubing is flattened upon cutting, open the end. Flattening of the tubing end may be avoided by using a tubing cutter that is sufficient for the diameter of the Tefzel tubing (0.125 in).

Each bullet will be 1 cm in length. Prepare a maximum of 26 bullets per 33 cm length of Tefzel tubing.

- 40** Use a 1 cm length piece of silicone adapter tubing to attach one end of the cut Tefzel tubing to a non-Luer Lok 10 mL syringe with its plunger. Retract the plunger by approximately 1 mL to facilitate later expulsion of liquid.

If necessary to identify different bullet preparations, label each length of Tefzel tubing with a small piece of tape near the adapter tubing. This location will allow later visualization of DNA-gold in the remaining length of the Tefzel tubing.

Luer Lok syringes make attaching the silicone adapter tubing slightly more difficult than non-Luer Lok syringes.

Prepare DNA-nanogold

Nanogold is complexed with DNA to form DNA-nanogold (Figure 2D). Per bullet, 57 μL of nanogold suspension will be mixed with a 15 μL volume of 10 μg of nucleic acid. The nucleic acid is the combined plasmid DNA and CpG oligodeoxynucleotide. If necessary to reduce the volume of the nucleic acid solution, the nucleic acid can be precipitated with sodium acetate and ethanol and then suspended in molecular grade water.

- 41** In a sterile polypropylene tube, combine the plasmid DNA and CpG oligodeoxynucleotide solutions in an amount that is sufficient for the number of bullets in the individually cut length of Tefzel tubing.
- Use a tube that will allow rapid vortexing, without the tube lid, of a total volume of 0.1 mL of solution per bullet. For example, for 26 bullets, a 2.6 mL volume can be vortexed in an open 15 mL tube.
- For tubes >1.5 mL, rapid transfer of the solutions in the following steps is facilitated by the use of snap-cap tubes.
- Immunization vectors are used at 1 µg per bullet. Adjuvant vector amounts vary (see Table 1).*
- Limit CpG oligodeoxynucleotide to 1 µg per bullet (see Troubleshooting).
- 42** To the nucleic acid mixture, add, per bullet, 57 µL of nanogold suspension.
- For example: for 26 bullets, combine the DNA solution (in a total volume of 390 µL) with 1482 µL of nanogold suspension.
- Before use, check that the stored nanogold solution is still deep wine red in color and clear.
- 43** Incubate the DNA-nanogold suspension at room temperature for 5 min.
- The suspension will turn from red to violet, indicating the formation of gold complexes of 300–400 nm size [Figure 2D; (Svarovsky et al., 2009)].*

Prepare DNA-gold micronanoplexes and bullets

DNA-gold micronanoplexes are formed by combining DNA-nanogold and microgold-PEI (Figure 2E).

- 44** In a polypropylene tube, prepare a fresh slurry of 167 mg/mL of microgold-PEI by combining, per bullet, 1 mg of lyophilized microgold-PEI with 6 µL of 0.1 M MES, pH 6.0.
- One slurry can be prepared for all of the cut Tefzel tubing lengths. In order to have sufficient slurry, weigh an amount of microgold-PEI that is ~20% larger than that required for the total number of bullets, and then add the appropriate volume of 0.1 M MES.
- For the slurry, use a tube size that allows vigorous vortexing of the slurry with an open lid.
- Quickly return the microgold-PEI to storage at 4°C under nitrogen gas and desiccant.
- Note that some of the microgold-PEI will adhere to the inner surfaces of tubes and pipet tips. This is normal.
- 45** While mixing the microgold-PEI slurry using rapid vortex, transfer by pipet an equivalent bullet-volume of the slurry to the DNA-nanogold suspension.

Immediately close the lid on the tube containing the combined microgold-PEI/DNA-nanogold and mix by vortex.

Mixing of the microgold-PEI slurry by vortex during pipetting is necessary because the microgold-PEI settles rapidly. In order to prevent clogging, rapidly pipet the slurry and use a pipet tip that has the largest possible size bore.

For example: to a DNA-nanogold suspension corresponding to 26 bullets, add 156 μL of microgold-PEI slurry.

- 46** Incubate the DNA-gold micronanoplex mixture for 10 min. During this incubation, mix by vortex every 3 min.

During this incubation, the supernatant becomes less colored, or colorless, due to complexation of the DNA-nanogold in the micronanoplex precipitate.

During this time, clean the Bio-Rad Tubing Cutter by wiping with ethanol and allow it to dry.

- 47** Centrifuge the micronanoplex mixture at $100 \times g$ for 1 min and discard ~95% of the supernatant using a pipet. Suspend the micronanoplex pellet in the remaining supernatant by gently pipetting. While vortexing the suspension, add 0.1 mL of ethanol per bullet, and then centrifuge again. Repeat this wash twice. At removal of the final supernatant, leave a minimal amount of supernatant in order to prevent drying out of the micronanoplex pellet.

Do not use centrifugation at $>100 \times g$ for micronanoplexes.

The initial supernatant can later be analyzed by ethidium-bromide-stained agarose gel electrophoresis in order to estimate the amount of DNA complexation into micronanoplexes.

Perform the next 5 steps in a chemical fume hood.

- 48** Use gentle pipetting to suspend the ethanol-washed DNA-gold micronanoplex pellet in, per bullet, 55 μL of 1-butanol.

When pipetting micronanoplexes, use a pipet tip with the largest possible size bore to prevent clogging.

For example: for 26 bullets, add 1430 μL of 1-butanol.

CAUTION: Butanol is flammable. Follow proper guidelines during use and disposal.

- 49** Use one hand to manipulate the syringe that is attached to the individual length of Tefzel tubing. Use the other hand to hold the micronanoplex tube and to secure the Tefzel tubing such that the open end of the tubing rests in the bottom of the micronanoplex suspension. Immediately after vortexing the micronanoplex suspension, rapidly draw the suspension into the Tefzel tubing by syringe aspiration. Immediately lay the tubing horizontally. Allow the filled tubing to rest for 5–10 min.

In order to allow even distribution of the micronanoplexes, it is important to rapidly draw the micronanoplex solution into the Tefzel tubing immediately after vortexing. Avoid repeated aspiration and ejection of the micronanoplex suspension, due to adherence of the micronanoplexes to the inner surface of the tubing.

Be careful to not pull the solution into the syringe barrel.

Do not disturb the Tefzel tubing during this incubation time, during which the micronanoplexes settle and adhere to the inner surface of the tubing.

Do not leave the butanol in contact with the micronanoplexes for more than 10 min due to the damaging effect of the butanol.

- 50** Gently pick up the Tefzel tubing. While holding the tubing horizontally, use the attached syringe to gradually expel the butanol at a constant rate from the open end of the tubing. Lay the tubing down horizontally. Discard the expelled butanol.

The expelled butanol solution should appear colorless. A pink solution indicates loss of DNA.

- 51** Dry the micronanoplexes in the tubing for 10–20 min by flowing helium through the tubing at a rate of 5 L/min, with the helium pressure regulator set at 20 psi. Upon drying, the gold turns a lighter shade. Stop the gas flow when all of the gold in the tubing turns a lighter shade.

The gold will first turn a lighter color (more yellowish) at the end of the tubing near the gas entry, followed by the remaining gold. To remove build-up of butanol at the other end of the tubing, gently touch that end to an absorbent pad to remove the liquid.

Do not over-dry the micronanoplexes as over-drying can inhibit release of the micronanoplexes upon firing. Under-drying also affects release: inspect the tubing for the absence of liquid droplets.

- 52** Enclose the Tefzel tubing by connecting the open ends with silicone adapter tubing until the time that the tubing can be cut.

- 53** Cut micronanoplex-containing Tefzel tubing into 1 cm length bullets using the Bio-Rad Tubing Cutter.

Avoid pushing the Tefzel tubing too hard into the hole of the Tubing Cutter, since the tubing can get pushed past the 1 cm mark.

For bullets containing different nucleic acid compositions, change the razor blade, wipe out the inside of the Tubing Cutter with ethanol and dry with paper towel.

- 54** Store bullets in polypropylene tubes containing desiccant and with protection from light. Use the bullets within 3 days.

For the storage tube, add 2–5 g of desiccant to a 15–50 mL tube and add a wadded Kimwipe for cushioning. Gently add the bullets using a pair of forceps, and top off the tube with sufficient Kimwipes to prevent the bullets from being jostled. Seal the lid tightly and wrap the lid with Parafilm.

BASIC PROTOCOL 2 GENE GUN IMMUNIZATION OF MICE

This protocol details immunization of mice using the gene gun (Figure 2F). The immunization process for micronanoplexes is the same as for conventionally prepared calcium/spermidine bullets. The immunization schedule described here is based on the schedule from (Hansen et al., 2016). Loading and firing of the gene gun is detailed in the *Helios Gene Gun System Instruction Manual* (Bio-Rad). Standard methods are also used for subsequent procedures, such as blood collection, isolation of B cells, and production of monoclonal antibodies. The immunization process is more easily carried out with two people, since two hands are typically needed to restrain the animal and to position the animal's ear. If necessary, mice can be individually marked. All animal procedures should be performed in accordance with approved institutional protocols and guidelines.

Materials

Reagents

Bullets from **BASIC PROTOCOL 1**

Forceps for handling bullets

Six- to eight-week-old female A/J or BALB/c mice (e.g., from Jackson Laboratory) housed on a 12 h dark/light schedule and provided food and water *ad libitum*; this age allows immunizations to be performed during the optimal age range of 8–12 weeks (Hu et al., 1990) following acclimatization and collection of pre-immunization serum samples

Equipment

Helios Gene Gun Kit (Bio-Rad #165-2411); includes gene gun, 5 O-rings, 5 Barrel Liners, 5 Cartridge Holders, Cartridge Extractor Tool, and 9 V battery

Helium Hose Assembly with Swagelok Quick-Connect fittings (Bio-Rad #165-2412)

Helium Regulator (Bio-Rad #165-2413)

Hearing protection

Genetic immunization of mice

1. Prior to Immunization Day 1: obtain a pre-immunization serum sample.
2. On the day of immunization: visually inspect bullets for even distribution of DNA-gold micronanoplexes before loading the gene gun.

Note that bullets that were formed near the ends of the uncut Tefzel tubing may have less DNA-gold.

Loading and firing of the gene gun is detailed in the Helios Gene Gun System Instruction Manual (Bio-Rad).

CAUTION: Wear hearing protection as the gene gun produces an intense, approximately 108 decibel sound upon firing at 400 psi.

3. Perform a test shot (350 psi) against a white piece of paper (do not use paper towel) by resting the Barrel Liner (front end) of the gene gun against the paper and firing.

A diffuse brown spot of approximately 7 mm diameter will be visible on the paper, indicating delivery of the DNA-gold micronanoplexes.

4. Check the spent bullet: the tubing should be mainly clear of visible gold.
5. Immunization Day 1: immunize mice in the ear skin pinnae using two bullets per mouse, with one bullet on each ear.

Each immunization consists of two bullets.

The gene gun Barrel Liner should be placed flush against the skin of the ear.

On alternate immunization days, switch between immunization of the front and back surfaces of the ear.

Alternative immunization sites may be used, such as the abdomen following hair removal [e.g., (Robinson and Pertmer, 2001)].

It is recommended to change the removable gene gun Barrel Liner between different DNA preparations.

The DNA-gold micronanoplexes will not penetrate a single layer of gloves.

6. Immunization Day 2 (or 3): repeat the immunization with 2 bullets per mouse.

The first two days of immunization constitute the double-prime.

The same batch of bullets can be used for both days of the double-prime.

7. Repeat the immunization every two weeks (the biweekly “boosts”) for a minimum of two boosts and a maximum of four boosts.

Note that for each boost, fresh batches of nanogold and bullets should be prepared.

8. Monitor serum antibody levels every two weeks, beginning with two weeks following the double-prime.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps unless noted.

0.1 M MES, pH 6.0

Dissolve 1.95 g of 2-(*N*-morpholino)ethanesulfonic acid (MES) in 90 mL of H₂O

Adjust to pH 6.0 with 10 M NaOH (*APPENDIX 2E*)

CAUTION: NaOH is corrosive. Wear the proper personal protective equipment.

Add H₂O to a final volume of 100 mL

Filter sterilize (0.2 µm) and store at room temperature

Solution should be colorless and free of precipitate

10% PEI, pH 9 (20 mL)

Weigh 2 g of branched polyethylenimine (PEI; average M_w ~25,000, average M_n ~10,000; e.g., Sigma #408727) into a 50 mL propylene tube

Add 15 mL of H₂O and vortex vigorously to dissolve

Adjust to pH 9 with concentrated HCl (37%, ACS grade; add approximately 1 mL) in order to protonate the amine groups

CAUTION: HCl is corrosive. Wear the proper personal protective equipment. Avoid breathing the vapor from concentrated HCl.

Add H₂O to a final volume of 20 mL and use fresh

508 mM HAuCl₄

Upon receipt, check that 1 g of hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄; 99.9%; e.g., Alfa Aesar #36400-03, or Sigma #520918-1G) is in a yellow, crystal form and is not wet

Suspend 1 g of HAuCl₄ in 5 mL of molecular grade water

CAUTION: HAuCl₄ causes severe skin burns and serious eye damage. Wear the proper personal protective equipment.

Top off the bottle with nitrogen gas, wrap lid with Parafilm, store at 4°C, and use within 6 months

COMMENTARY

Background Information

Biolistic immunization for antibody production—In 1990 it was first shown that protein could be expressed *in vivo* upon injection of naked DNA into mouse muscle (Wolff et al., 1990). Soon after it was clear that DNA delivery leads to a protective immune response that includes the appearance of antigen-specific antibodies (Tang et al., 1992; Robinson et al., 1993) and also allows isolation of monoclonal antibodies (Barry et al., 1994). The Tang et al. (1992) study used a biolistic device that included a vacuum chamber to deliver DNA/calcium/spermidine-coated micron particles to the mouse ear (Tang et al., 1992). Subsequently, the more portable and vacuum-free Helios gene gun device was developed.

In addition to biolistic methods, DNA can be delivered *in vivo* by simple needle injection of naked DNA and by electroporation-based methods. Whatever the route, method development has mainly focused on vaccine studies. Several comparative studies indicate that these diverse delivery methods vary greatly in the types of immune responses and in the efficiency of titer formation (Feltquate et al., 1997; Wang et al., 2008), likely due in part to different cellular uptake mechanisms (Tüting, 1999). All of these studies involved soluble proteins or the soluble domains of membrane-anchored proteins.

Genetic immunization methods for antibody production against membrane proteins—Many published studies describing antibody production for membrane proteins that used genetic immunization have included boosts with some form of the protein, such as purified protein or cells expressing the protein (Sanders et al., 2002; Koch-Nolte et al., 2005; Lee et al., 2014). However, there are several reports for individual proteins that evidently used only DNA as immunogen. While some of these reports used proprietary methods (Elagoz et al., 2004; Fofana et al., 2013), others provided sufficient detail to indicate successful approaches. Tymciu et al. (2002) obtained conformation-dependent antibodies and measurable titers at a 1:200 serum dilution for the neurokinin-1 GPCR using the gene gun and the pcDNA3.1 vector, in the absence of fusion sequences or adjuvants (Tymciu et al., 2002). Naito et al. (2011) raised antibodies that recognized the natively folded human nephrin transmembrane protein by using the gene gun and expression driven by a CMV promoter/intron, but only upon inclusion of the nephrin N-terminal membrane-targeting signal sequence (Naito et al., 2011). Antibodies that recognized the native human endothelin receptor GPCR were obtained with a C-terminal GroEL fusion under a CAG promoter (CMV enhancer fused to the chicken beta-actin promoter) combined with intramuscular injection and electroporation (Fujimoto et al., 2012). Genentech isolated conformational monoclonal antibodies for the 12-transmembrane multi-drug resistant protein 4 (MRP4) using immunization by hydrodynamic tail vein delivery of a CMV promoter vector plus adjuvant vectors expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) and fetal liver tyrosine kinase 3 ligand [Flt3L; (Hazen et al., 2014)]. These authors also noted prolonged *in vivo* expression of a fusion of MRP4-luciferase upon substituting the CAG promoter. It is exciting to see genetic immunization methods being specifically developed for the generation of conformation-dependent and functional antibodies against membrane-embedded proteins of high therapeutic interest.

Method comparison—With respect to membrane protein antigens, there is yet no published comparison of antibody quality or characteristics (e.g., affinity, specificity, or percent conformation-dependence) between the various DNA-based methods or using different immunization sites (ear; abdomen; etc.) (Liu et al., 2016). The same can be said about comparison of genetic immunization with the other major non-DNA methods of antibody generation: needle injection of purified protein or antigen-expressing cells, and *in vitro* display. Due to the unique *in vivo* presentation afforded by biolistic immunization, further method development is anticipated.

DNA-gold micronanoplexes—Two reports by Svarovsky et al. (2008, 2009) improved microparticle efficiencies by applying self-assembled monolayer chemistry and adding

submicron gold. “PEI-gold microparticles” were a forerunner of microgold-PEI and involved sequential surface modification with (2–mercaptopropionyl)glycine (instead of MUDA) and PEI (Svarovsky et al., 2008). Compared to calcium/spermidine micron gold, PEI-gold microparticles were more stable, as they showed less shearing, and yielded at least a two-fold higher DNA binding and consequent higher luciferase activity. The PEI-gold microparticles also supported delivery of small interfering RNAs (siRNA) as shown by luciferase knockdown (Svarovsky et al., 2008). Upon development of the DNA-gold micronanoplex method, MUDA was substituted for (2–mercaptopropionyl)glycine, nanogold was added, and the micronanoplexes were characterized by electron microscopy (Svarovsky et al., 2009). Compared to calcium/spermidine micron gold, the micronanoplexes also showed an 11–fold higher DNA binding and an increase in luciferase expression *in vivo* (Svarovsky et al., 2009). DNA-gold micronanoplexes were first applied to antibody production in our recent report (Hansen et al., 2016).

DNA-gold micronanoplex generation has not yet been combined with the Bio-Rad Tubing Prep Station (Bio-Rad #165-2418) that is described in the *Helios Gene Gun System Instruction Manual* (Bio-Rad). This Prep Station is available separately (#165-2418) or as part of a complete package with the gene gun (#165-2431). The major differences with the protocol described in this unit are that the Tubing Prep Station involves rotation of the Tefzel tubing to achieve coating of the full inner surface of the tubing. Without the need for tubing rotation, the protocol described in this unit allows simultaneous drying of multiple tubes upon applying a multi-port gas manifold capable of a 5 L/min flowrate (e.g., from machine shop).

Critical Parameters

Animal considerations—In our recent report (Hansen et al., 2016), A/J strain mice had been chosen based on previous experimental data showing strong antibody production upon biolistic immunization with calcium/spermidine micron gold (Li et al., 2006). Both A/J and BALB/c strains are suitable for production of monoclonal antibodies (Hansen et al., 2016). Anesthetic is not required during gene gun bombardment. Following delivery, there should be no apparent damage to the skin. Although the immunization process does not generally yield adverse effects, the health of the mice should be monitored since there may be unanticipated effects that are antigen-specific.

Analyses of the resulting antibodies—To identify conformation-dependent and functional antibodies, antigen quality is critical. For antibody analyses, it is recommended to a native ELISA format, such as indirect immobilization of the membrane protein *via* protein tags (Lim et al., 2011; Mir et al., 2015). Direct binding of membrane protein antigen to the surfaces of ELISA plates is expected to distort the protein structure (Mir et al., 2015).

Troubleshooting

Bullet quality—Quality of the bullets can be affected by several parameters. Poor quality bullets will result if micronanoplexes are observed to flake off of the bullets before firing or that fail to release upon firing. These problems can be caused by excessive humidity, by over-drying or under-drying the bullets under helium gas flow, by an inappropriate ratio of

DNA-nanogold:microgold-PEI, or by too much CpG oligodeoxynucleotide (>1 µg/bullet). High CpG oligonucleotide amounts (>1 µg/bullet) cause stickiness of DNA-gold. We have determined that this stickiness increases with the amount of CpG such that complete DNA-gold release was obtained at 1 µg/bullet, partial release at 2.5 µg/bullet, and no release at 5 µg/bullet (at 350 psi). Lack of micronanoplex release may be overcome by increasing the gene gun gas pressure up to a maximum of 600 psi. The bullet production process is also highly impacted by the quality of the chemicals. Upon receipt, check MUDA, NHS, EDC, cysteamine and NaBH₄ for powder/granular form and for a lack of dampness. For storage, top off MUDA, NHS and EDC chemicals with nitrogen gas and wrap lids with Parafilm. This protocol also identifies the expected changes in color and sample appearance at several steps. Due to possible uneven distribution of the micronanoplexes on the tubing, it is recommended that more bullets be prepared than the number needed.

Positive control vector for immunization—A positive control immunization vector that expresses the highly immunogenic FopA/FTT0583 membrane protein from *Francisella tularensis* (Hansen et al., 2016) is available through the DNASU Plasmid Repository as plasmid FTT0583 in pCMVi-LSrCOMPTT (Table 1). Plasmids for expressing the FopA/FTT0583 protein are also available and are described in (Hansen et al., 2016).

Anticipated Results

We recently reported the first description of the efficiency of any DNA immunization approach targeting membrane proteins by applying a set of proteins (n = 17) from two Biosafety Level 3 pathogens (Hansen et al., 2016). For this structurally diverse set, we demonstrated a 71% success rate for antibody production, which approached the 84% rate (n = 100) previously described for soluble proteins (Chambers and Johnston, 2003). Success was scored by positive ELISA and Western at 1:500 serum dilution for at least 1 out of 5 mice (Hansen et al., 2016). For membrane protein antigens that have had previous reports of immunogenicity in infected hosts, measurable antibody titers should be apparent in all mice within two weeks following the first boost. Highly immunogenic proteins may yield positive responses at 1:10,000 serum dilution after two boosts (Hansen et al., 2016). After four boosts, poor immunogens may yield positive titers at a lower serum dilution (1:500) and in only some of the mice.

Time Considerations

The design and generation of immunization plasmid constructs can take three weeks. Bullet production requires three days: one day for preparation of solutions (4 hr); one day for microgold-PEI preparation (8 hr), plus an overnight lyophilization step; and one day for the remaining bullet production steps (nanogold; DNA-nanogold; DNA-gold micronanoplexes; and bullets; 5 hr). The immunization step is rapid, requiring approximately 15 min per cage, and so can be completed on the third day. Once an acclimatization period has been completed for the mice, the immunization schedule may require 6–10 weeks depending on whether 2–4 boosts are applied. The entire process therefore requires 8–12 weeks.

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FIGURE 1.

Genetic immunization vector pCMVi-LSrCOMPTT. The vector as shown contains the encoded membrane protein antigen (target ORF). The empty parent vector, without the target ORF, contains the following unique restriction sites located between the tetanus toxoid epitopes (TT) and the transcription terminator (Ter): *Bgl*II, *Kpn*I, *Mlu*I, *Cla*I, *Hind*III, *Sbf*I, *Xba*I, *Bam*HI and *Sma*I. This vector confers ampicillin-resistance upon propagation in *E. coli*. Abbreviations: P_{CMV}, cytomegalovirus immediate-early promoter; LS, leader sequence; r, randomly-generated immunogenic peptide; COMP, cartilage oligomeric matrix protein; TT, tetanus toxoid epitopes; ORF, open reading frame; hGH Ter, human growth hormone transcription terminator.

FIGURE 2.

Overview of DNA-gold micronanoplex bullet production. DNA-gold micronanoplexes are complexes of two differently-sized gold particles: microgold-PEI, which are micron-sized gold particles that are coated with polyethylenimine (Svarovsky et al., 2008), and DNA-nanogold, which are nanometer-sized gold particles that are generated upon modification with cysteamine and DNA (Svarovsky et al., 2009; Hansen et al., 2016). See the figure for chemical abbreviations. (A) MUDA allows alkanethiol functionalization of the micron gold surface. (B) NHS and EDC activate the carboxylic acid groups of MUDA. This activation will allow carbodiimide crosslinking with the primary amines (Frey and Corn, 1996) of PEI to form microgold-PEI (Svarovsky et al., 2008). (C) Molecular gold is modified with cysteamine to form nanogold, which are particles of ~36 nm diameter (Svarovsky et al., 2009). These larger gold particles cause the solution to turn from yellow to a deep wine red. (D) DNA is complexed with nanogold to form DNA-nanogold, which forms particles of ~300–400 nm diameter (Svarovsky et al., 2009). The larger gold particles cause the solution to turn from deep wine red to a violet color. (E) DNA-nanogold is combined with microgold-PEI to form DNA-gold micronanoplexes. Here, the positively charged microgold complexes with the negatively-charged DNA. (F) Gene gun immunization using DNA-gold

micronanoplex bullets yields a serum antibody response following *in vivo* expression of the membrane protein antigen. The shaded inset is a schematic of the expressed membrane protein in a lipid bilayer. Images were adapted from (Hansen et al., 2016).

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TABLE 1

Plasmid DNA vectors for *in vivo* expression of membrane protein antigen and adjuvants

Vector	Amount/bullet	DNASU Plasmid ID ^a or Source	Reference
<i>Empty vectors for encoding the antigen (see Strategic Planning)</i>			
pCMVi-LSrCOMPTT	1 µg	EvNO00629005	(Hansen et al., 2016)
pCMVi-LS	1 µg	EvNO00631283	(Sykes and Johnston, 1999; Hansen et al., 2016)
<i>Antigen-encoding vector (optional positive control)</i>			
FTT0583 in pCMVi-LSrCOMPTT	1 µg	FtCD00665310	(Hansen et al., 2016)
<i>Vectors expressing adjuvants (optional; see Strategic Planning)</i>			
pCMVi-LS-LTA-R192G	21 ng ^b	EcCD00697199	(Whitlock et al., 2011; Hansen et al., 2016)
pCMVi-LS-LTB	104 ng ^b	EcCD00697200	(Whitlock et al., 2011; Hansen et al., 2016)
pCMVi-mGMCSF ^c	250 ng	S.A. Johnston	(Chambers and Johnston, 2003)
pCMVi-Flt3L ^d	250 ng	S.A. Johnston	(Chambers and Johnston, 2003)

^aThe DNASU Plasmid Repository can be found at dnasu.org (Seiler et al., 2014).

^bThese amounts yield an equimolar ratio of the genes encoding the enterotoxigenic *E. coli* heat-labile enterotoxin subunits A (LTA; inactive R192G mutant) and B (LTB).

^cmGMCSF, mouse granulocyte-macrophage colony-stimulating factor (GenBank accession number CAA26820.1).

^dFlt3L, mouse fetal liver tyrosine kinase 3 ligand (GenBank accession number AAA39436.1).