Comparative Measurement of Cell-Mediated Immune Responses of Swine to the M and N Proteins of Porcine Reproductive and Respiratory Syndrome Virus

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Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is an enveloped virus that belongs to the genus Arterivirus, the family Arteriviridae, and the order Nidovirales. PRRSV has a single-stranded positive-sense RNA genome composed of nine open reading frames (ORFs) (5). PRRSV is currently recognized as the pathogen responsible for the greatest economic losses in the swine industry worldwide. PRRSV induces late-term reproductive failure in sows and pneumonia in young pigs (30, 41). The establishment of persistent infection is one of the principal characteristics of PRRSV (42). The delayed induction of both T cell-mediated immunity and neutralizing antibodies is assumed to contribute to persistent PRRS infection (8, 26). The control of PRRSV by vaccination is challenging because of the significant antigenic diversity known to exist among all strains of this RNA virus and the resultant insufficient generation of protective immunity toward heterologous PRRS strains (18). The correlates of the protection indicators of broad reactive immunity attainable by vaccination have yet to be well-defined (22).

Serum neutralizing antibodies may be one of the factors contributing to protection against PRRSV (22). The GP5 and GP4 proteins are representative antigens that contribute to the induction of neutralizing antibodies (13, 27). However, it has become evident that T cell-mediated immunity is essential for effective protection against PRRSV (25, 47). The increased cellular immunity was significantly related to the reduced clinical symptoms in PRRSV-infected piglets and sows (17, 23). Analysis of the T-cell responses to the structural proteins of PRRSV indicated that the M protein may perform a major role in the induction of cellular immunity in infected pigs (2). When the M protein was fused with GP5, it induced stronger cell-mediated immunity compared to that achieved with the M protein alone (16, 46). By way of contrast, only a few papers have suggested that the N protein may have the capacity to induce cell-mediated immunity and to reduce the viral loads in infected animals (19, 29). In order to gain a better understanding of the nature of immunity to PRRSV, further comparative analyses of cell-mediated immunity against the M and N proteins of PRRSV (the PRRSV-M and PRRSV-N proteins, respectively) should be conducted.

Although a few lines of inbred miniature pigs which express the homologous major histocompatibility complex (MHC) molecules exist (6, 28), the majority of outbred conventional pigs harbor polymorphic MHC molecules on their cells. That is...
the primary reason that it has proven difficult to conduct a proper analysis of MHC-restricted T-cell immune responses in pigs immunized with vaccines or infected with the wild-type viruses. Therefore, the development of MHC-matched autologous antigen-presenting cells (APCs) is important for assessment of the T-cell-mediated immune responses in pigs (20).

The generation of autologous APCs with primary cells is also relatively difficult, owing to the insufficient transfection or expression efficacy of the viral genes delivered into them (3). The transactivator of transcription (TAT) protein of human immunodeficiency virus (HIV) type 1 (HIV-1) harbors a protein transduction domain (PTD) (12, 14). It has previously been determined that TAT-conjugated macromolecules can efficiently be delivered into a variety of mammalian cells (3, 31). APCs transfected with the TAT conjugated with viral antigens could induce T-cell specific immune responses to those antigens (32, 36, 38). The well-described ability of TAT fusion proteins to rapidly transduce proteins into many primary mammalian cells suggests that this property might be exploited to develop autologous APCs displaying viral antigens in outbred animals (3).

In the study described here, we immunized conventional pigs with two plasmid DNA constructs encoding the PRRSV M and N proteins that were fused with the porcine granulocyte-macrophage colony-stimulating factor (GM-CSF). This system was useful for analysis of the immune responses very specific to individual antigens in the vaccinated pigs. The property of TAT-mediated protein transduction was newly applied in an effort to generate MH-C-matched and autologous primary APCs in pigs. The APCs were used to stimulate memory T-cell populations in peripheral blood mononuclear cells (PBMCs) isolated from the immunized pigs. Different features of the T-cell-mediated immune responses induced by the M and N proteins of PRRSV and the application of the protocol to generate autologous APC are discussed herein.

### MATERIALS AND METHODS

**Animals.** A total of 10 conventionally reared 4-week-old pigs were obtained from a PRRS-free herd. Their PRRSV-negative status was confirmed prior to immunization.

**Cloning of ORFs 6 and 7 of PRRSV into cloning and expression vectors.** PRRSV strain LMY (GenBank accession number DQ473474), the representative PRRSV strain isolated in South Korea, was used to clone orf6 and orf7. It was provided by the National Veterinary Research Quarantine Service (NVRQS), Anyang, South Korea. Marc-145 cells were infected with 10^5.7 50% tissue culture infective doses/ml PRRSV. When complete cytopathic effects (CPEs) were apparent at day 5 after infection, viral RNA was purified from the tissue culture supernatants by use of a QIAamp viral RNA minikit (Qiagen, Valencia, CA), in accordance with the manufacturer's instructions. The single-stranded cDNA was synthesized in a tube containing 20 μl of reaction reagents, including 1 μl of oligo(dT) primer, 4 μl of 5X buffer, 2 μl of 0.1 M dithiothreitol, 1 μl of 10 mM deoxynucleoside triphosphates, 1 μl of DNaseI, and 1 μl of Moloney murine leukemia virus (MMLV) RT (4 units/μl; Qiagen) at 37°C for 1.5 h. The single-stranded cDNA was synthesized in a tube containing 20 μl of reaction reagents, including 5 μg of viral RNA, 1 μg of oligo(dT) primer, 4 μl of 5X buffer, 2 μl of 0.1 M dithiothreitol, 1 μl of 10 mM dNTPs, 1 μl of RNasin, and 1 μl of MMLV RT (4 units/μl; Qiagen) at 37°C for 1.5 h. The entire orf6 and orf7 of the cDNA, including two restriction enzyme sites for EcoRI and EcoRV at the 5’ and 3’ ends, respectively, was amplified by PCR with primers PRRSV-M F2 and PRRSV-M R2 (Table 1) and the cDNA. Similarly, the entire orf7, including two restriction enzyme sites for EcoRI and EcoRV at the 5’ and 3’ ends, respectively, was amplified by PCR with primers PRRSV-N F2 and PRRSV-N R2 (Table 1) and the cDNA.

**Generation of DCs.** Porcine myeloid-derived dendritic cells (DCs) were generated as described previously but with some modifications (15, 39). In brief, PBMCs were isolated from heparin-treated whole-blood cells of each pig by 30 min of centrifugation at 400 g. After 4 h of culture, the bacterial cells were collected by centrifugation. The recombinant proteins were purified through a nickel column, in accordance with the manufacturer's protocols. The identities of purified PRRSV-M, PRRSV-N, and PRRSV-N were confirmed by Western blotting. The proteins blotted onto the nitrocellulose membranes (Whatman, United Kingdom) were exposed to porcine serum acquired from a PRRSV-infected pig. The membranes were then exposed to secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG (Southern BioTech). The protein-specific bands were identified by color development with 3,3'-diaminobenzidine (DAB, Pierce).

**Expression of recombinant proteins in E. coli.** E. coli (M 15; Qiagen) colonies harboring the pQE30-PRRSV-M, pQE30-PRRSV-N, and pQE30-PRRSV-N clones were inoculated to 20 ml of LB broth containing 100 μg/ml of ampicillin (Sigma Aldrich, St. Louis, MO) and 25 μg/ml of kanamycin (Sigma Aldrich) and incubated for 12 h at 37°C. The bacterial cultures were then inoculated into 1,000 ml of (rash LB broth. When the optical density (OD) value (600 nm) of the bacterial cultures reached 0.6, iso-propl-b-β-thiolactosaminide (Gene All, South Korea) was added to a final concentration of 1 mM. After 4 h of culture, the bacterial cells were collected by centrifugation. The recombinant proteins were purified through a nickel column, in accordance with the manufacturer's protocols. The identities of purified PRRSV-M, PRRSV-N, and PRRSV-N were confirmed by Western blotting. The proteins blotted onto the nitrocellulose membranes (Whatman, United Kingdom) were exposed to porcine serum acquired from a PRRSV-infected pig. The membranes were then exposed to secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG (Southern BioTech). The protein-specific bands were identified by color development with 3,3'-diaminobenzidine (DAB, Pierce).

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Construction of mammalian expression vector DNAs. Porcine PBMCs were cultured for 2 days in the presence of 5 μg/ml of concanavalin A (ConA; Sigma Aldrich). Total cellular RNA was extracted from the cells with an RNeasy minikit (Qiagen), in accordance with the manufacturer’s instructions. After the synthesis of cDNA with oligo(dT) primer and cellular RNA by MMLV RT, the GM-CSF gene was amplified with primer GM-CSF FP harboring a Kozak sequence and primer GM-CSF RP (Table 1). PCR was conducted for 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. The PCR product was ligated into the mammalian expression vector pcDNA3.1 (Invitrogen), in accordance with the manufacturer’s procedures. The clone was designated pcDNA3.1-GM-CSF. DNA vaccines were constructed by conjugating the porcine GM-CSF gene with the PRRSV-M and PRRSV-N genes in the pcDNA3.1 vector. In brief, pCR2.1-PRRSV-M and pCR2.1-PRRSV-N were digested with EcoRI and EcoRV. The clones were designated pcDNA3.1-GM-CSF-PRRSV-M and pcDNA3.1-GM-CSF-PRRSV-N, respectively.

### Expression of GM-CSF-PRRSV-M and GM-CSF-PRRSV-N genes in mammalian cells.

3T3 cells adjusted to 5 × 10⁶/ml were cultured for 1 day in Dulbecco modified Eagle medium (DMEM) (Welgene, South Korea) supplemented with 10% fetal bovine serum without antibiotics in six-well plates. The transfection reagents were prepared in accordance with the manufacturer’s instructions. In brief, 4.0 μg of plasmid DNA—either pcDNA3.1-GM-CSF-PRRSV-M or pcDNA3.1-GM-CSF-PRRSV-N DNA—was dissolved in 250 μl of total RNA with 1 μg of DNA was removed from the RNA by treating 1 U/μl of DNase I (1 U/μl; Invitrogen) in 250 μl of DMEM, and the DNA bands containing the PRRSV-M and PRRSV-N genes were purified. The PRRSV-M and PRRSV-N genes were ligated into pcDNA3.1-GM-CSF, which had been digested with EcoRI and EcoRV. The clones were designated pcDNA3.1-GM-CSF-PRRSV-M and pcDNA3.1-GM-CSF-PRRSV-N, respectively.

| Vector | Primer name | DNA sequence (5’ → 3’) | Product size (bp) |
|--------|-------------|------------------------|------------------|
| pCR2.1-TOPO | PRRSV-M FP1 | GAA TTC ATG GGC TGG TCG TCC TTA GAT GAC <i>a</i> | 537 |
| pCR2.1-TOPO | PRRSV-M RP1 | GAT ATC TTA TTT GGC ATA TTT AAC AGG <i>a</i> | 537 |
| pQE30-UA | PRRSV-N FP1 | GAA TTC ATG CCA AAT AAC AAG GGC AAG CA <i>a</i> | 384 |
| pQE30-UA | PRRSV-N RP1 | GAT ATC TCA GGC TGA GGG TGA TGC TGT G <i>d</i> | 370 |
| pQE30-UA | PRRSV-N FP2 | TAC TCA GGC ATA GAA ACC | 270 |
| pQE30-UA | PRRSV-N RP2 | TTA TTT GGC ATA TTT AAC AGG | 270 |
| pCR2.1-TOPO | PRRSV-M FP3 | CTC GAG ATG CCA AAT AAC AAG GGC AAG CA <i>a</i> | 384 |
| pCR2.1-TOPO | PRRSV-N FP3 | CTC AGA TCA GGC TGA GGG TGA TGC TGT G <i>d</i> | 370 |
| pQE30 | TAT-PRRSV-M FP | GGA TCC GGC AGG AAG AGG CGG AGA CAG CCA AGA CCT CCT CCA TGC GCA TTG AGG AGG TCT TCG TCT CGT CCT CTT CTT GCC | 42 |
| pQE30 | TAT-PRRSV-M RP | GTC GAC TTA TTT GGC ATA TTT AAC <i>c</i> | 324 |
| pQE30-UA | TAT-PRRSV-N FP1 | GGC AGG AAG AAG CCG AGA CAG CCA AGA CCT | 414 |
| pQE30-UA | TAT-PRRSV-N RP1 | TCT CAA TGC TAC TCA GCC ATTA GAA ACC | 324 |
| pcDNA3.1 | GM-CSF FP | GCC GCC GCC ATG GGG CTG CAG AAC CTG CTT CCT CTT CCC CCA GCA A | 447 |

<i>a</i> Italics indicate the EcoRI restriction enzyme site.
<i>b</i> Italics indicate the EcoRV restriction enzyme site.
<i>c</i> Italics indicate the SalI restriction enzyme site.
<i>d</i> Italics indicate the XbaI restriction enzyme site.
<i>e</i> Italics indicate the BamHI restriction enzyme site.
<i>f</i> Italics indicate the Sall restriction enzyme site.
<i>g</i> Italics indicate the XhoI restriction enzyme site.
<i>h</i> Italics indicate the EcoRV restriction enzyme site.
Immunization of pigs with plasmid DNAs and determination of antibody titers. A total of 10 pigs were employed in this study. Two immunization groups, composed of three pigs per group, were intramuscularly immunized with 500 μg of pcDNA3.1-GM-CSF-PRRSV-M and pcDNA3.1-GM-CSF-PRRSV-N, respectively. The plasmid DNAs were mixed with the adjuvant dimethyldioctadecylammonium bromide (DDA; Sigma Aldrich) prior to injection into the pigs, as described in the report of another study (37). Four pigs were injected with phosphate-buffered saline and utilized as a nonimmunized control group. The plasmid DNAs were administered a total of 8 times on days 0, 7, 14, 21, 28, 35, 100, and 193. Sera were collected from the immunized and control pigs prior to each injection. The PRRSV-specific antibody titers in the sera were determined by ELISA. In brief, 5 μg/ml of the recombinant PRRSV-M and PRRSV-N proteins were coated onto microwell plates overnight. The serum samples, diluted 50-fold, were added to the respective antigens and incubated for 2 h at 37°C. The 5,000-fold-diluted goat anti-porcine IgG-HRP was added to the plates. After 20 min of incubation at 37°C, tetramethylbenzidine substrate was added to develop the color and H2SO4 stop solution was added to the reaction mixture. The OD values at 450 nm were determined with an automatic ELISA reader (Tecan Sunrise, Switzerland).

Assessment of T-cell proliferation and cytokine production. All experiments designed to evaluate T-cell proliferation and cytokine synthesis were conducted in 96-well flat-bottom plates (SPL, South Korea) with MHC-matched autologous APCs and effector T cells for each pig. T-cell-containing PBMCs were prepared from nonimmunized and immunized pigs 14 days after the final vaccination. The PBMCs and monocytoid-derived DCs were used as two different types of APCs to deliver PRRSV-M or PRRSV-N antigens to effector T cells. The PBMCs, which had been stimulated for 5 days with ConA (5 μg/ml), were treated with TAT-PRRSV-M or TAT-PRRSV-N protein to use them as the APCs. The DCs were used as APCs after the immature DCs were treated with TNF-α (250 U/ml) for 16 h to make them mature. The TAT-PRRSV-M or TAT-PRRSV-N protein (5 μg/ml) was also added to the immature DCs during the maturation period. Those antigen-presenting PBMCs and DCs were then treated for 1 h with mitomycin C (10 μg/ml; Sigma Aldrich). The APCs were added to effector T cells at effector T-cell to APC (E/A) ratios of 5:1 (5 × 106 effector cells/ml) and the cells were incubated for 3 days. Positive control cells were stimulated with 5 μg/ml of ConA and incubated under conditions identical to those employed for the APC-stimulated cells. T-cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Sigma Aldrich), in accordance with the manufacturer's instructions. In brief, 10 μl of MTT reagent (5 mg/ml) was added to each well of the plate, and the plate was incubated for 3 h in a cell culture incubator. One hundred microliters of stop solution (20% SDS in 50% dimethyl formamide) was added to each well, and the plate was incubated for 1 h at room temperature. The OD values of each well were determined with an ELISA reader at 540 nm. The amounts of porcine gamma interferon (IFN-γ), IL-6, and IL-10 in the cell culture supernatants collected from the antigen-stimulated T cells were determined with conventional ELISA kits (R&D Systems), in accordance with the manufacturer's instructions.

Statistical analysis. T-cell proliferation and cytokine production were measured at least three times, in triplicate, per pig. The significance of the difference in the experimental data between individual pigs in the vaccinated and the control groups was determined by Student’s t test with the Instat (version 3.0) program (GraphPad Software, San Diego, CA).

RESULTS

Expression of TAT-conjugated PRRSV-M and PRRSV-N proteins. As the M protein of PRRSV has transmembrane regions that hamper protein expression in E. coli, we deleted the transmembrane regions at the 5’ end, which encompassed nucleotides 1 to 255 (amino acids 1 to 85). This deletion made possible the expression of the remaining PRRSV-M region in E. coli (data not shown). The fusion gene of the TAT and transmembrane region-deleted orf6 of PRRSV was cloned into the E. coli expression vector. By way of contrast, the entire N gene of PRRSV was cloned into the E. coli expression vector as a fused form with the TAT gene (Fig. 1A). The recombinant proteins of TAT-PRRSV-M and TAT-PRRSV-N were expressed in E. coli transformed with the pQE30-TAT-PRRSV-M and pQE30-UA-TAT-PRRSV-N vectors, respectively. The pure TAT fused PRRSV-M and PRRSV-N proteins were identified by Western blotting to be 11.4- and 15.1-kDa proteins, respectively (Fig. 2A and B).

Generation of DCs. The immature monocyte-derived DCs were generated by culturing the PBMCs for 6 days in tissue culture medium supplemented with porcine GM-CSF and IL-4. The immature DCs expressed relatively low levels of the typical DC markers. The levels of expression of the DC markers in the immature DCs were 85% for MHC class I molecules, 35% for MHC class II molecules, 38.5% for CD172a molecules, and 51% for CD80/CD86 molecules (Fig. 3A). The immature DCs were induced to mature by treatment with TNF-α. Such treatment induced substantially higher levels of expression of all of the DC markers for which the immature DCs were assessed. The levels of expression of the DC markers in the mature DCs were 98% for MHC class I molecules, 96% for MHC class II molecules, 95% for CD80/CD86 molecules and 99% for CD80/CD86 molecules (Fig. 3B). The highly enhanced

FIG. 1. Cloning of viral genes into protein expression vectors. (A) The recombinant TAT-PRRSV-M and TAT-PRRSV-N genes were cloned into the pDrive and pCR2.1 cloning vectors, respectively. Once they were cloned into the cloning vectors, the insert DNAs were subcloned into the pQE30 and pQE30-UA E. coli expression vectors. (B) The porcine GM-CSF gene was cloned into the pcDNA3.1 mammalian protein expression vector. orf6 and orf7 of PRRSV cloned in the pCR2.1 vector were subcloned downstream of the GM-CSF gene.

FIG. 2. Expression of the recombinant proteins in E. coli. The recombinant TAT-PRRSV-M (A) and TAT-PRRSV-N (B) proteins were expressed in E. coli after transformation with the pQE30-TAT-PRRSV-M and the pQE30-UA-TAT-PRRSV-N vectors, respectively. The identities of the products expressed were confirmed by Western blotting with porcine serum obtained from a PRRSV-infected pig, followed by Western blotting with HRP-conjugated goat anti-porcine IgG. The protein bands were identified by color development involving DAB. Lanes 1, standard protein markers.
expression of the MHC and costimulatory molecules in the mature DCs suggested that they might prove useful as APCs to stimulate antigen-specific T cells.

**Transduction of TAT-fused PRRSV-M and PRRSV-N proteins into porcine cells.** The TAT-conjugated PRRSV-M and PRRSV-N proteins were transduced into PK15 cells. After transduction, the TAT-PRRSV-M and TAT-PRRSV-N proteins in the cell lysates were shown by Western blotting to have the predicted sizes of 11.4 and 15.1 kDa, respectively (Fig. 4A and B). The TAT-conjugated proteins could also transduce the primary porcine PBMCs and DCs (data not shown). These results clearly demonstrate that the TAT-conjugated PRRSV-M and PRRSV-N proteins can enter the primary cells as well as the cells of the established cell line. The data also suggest that the MHC-matched autologous primary cells, such as DCs and PBMCs, transduced with the TAT-conjugated PRRSV-M and PRRSV-N proteins could be used as APCs to stimulate antigen-specific T cells.

**Expression of PRRSV-M and PRRSV-N proteins in mammalian cells.** The entire PRRSV-M and PRRSV-N genes were subcloned downstream of the porcine GM-CSF gene in the mammalian expression vector pcDNA3.1 (Fig. 1B). The expression of the recombinant genes and proteins in 3T3 cells was identified after transfection with the pcDNA3.1-GM-CSF-PRRSV-M and pcDNA3.1-GM-CSF-PRRSV-N vectors by the RT-PCR and Western blotting methods, respectively. Expression of both the GM-CSF-fused PRRSV-M and PRRSV-N recombinant genes was identified. The recombinant GM-CSF-PRRSV-M and GM-CSF-PRRSV-N genes were identified as 978- and 825-bp RT-PCR products, respectively (Fig. 5A and B). The expression of both the PRRSV-M and PRRSV-N proteins fused with GM-CSF in the transfected cells was identified by Western blotting. The recombinant GM-CSF-PRRSV-M and GM-CSF-PRRSV-N proteins were found to be 35.9 and 30.8 kDa, respectively, by using the convalescent-phase serum from pigs infected with PRRSV (Fig. 5C and D). When the cell lysates were analyzed with the monoclonal antibody specific for porcine GM-CSF, protein products of the same size were detected (data not shown). These results show that the vectors constructed to express the PRRSV-M and PRRSV-N proteins as GM-CSF-fused recombinant proteins functioned properly in the mammalian cells. Therefore, we confirmed that the protein expression vectors constructed in this study could be used to immunize pigs.

**Antibody responses in pigs immunized with plasmids.** Antibody titers to the M and N proteins of PRRSV in the vaccinated and control pigs were determined by ELISA. The recombinant M and N proteins expressed from *E. coli* transformed with the pQE30-UA-PRRSV-M2 and pQE30-UA-PRRSV-N2 vectors were used as coating antigens in the ELISA. Three pigs immunized with pcDNA3.1-GM-CSF-PRRSV-M did not generate PRRSV-M protein-specific antibody (Fig. 6A). Until the pigs were immunized a total of 8 times, the antibody levels did not differ significantly from those noted in the control pigs. However, the other three pigs immunized with pcDNA3.1-GM-CSF-PRRSV-N generated PRRSV-N protein-
specific antibody (Fig. 6B). After the fourth immunization with the corresponding plasmid DNA, the production of antibody to the N antigen was apparent in the immunized pigs. Thereafter, the level of antibody production increased steadily after the boosting injection of the plasmid. The antibody titers measured in the two immunized pigs were at least 4 to 5 times higher than those identified in the control pigs. On the other hand, the antibody level measured in one immunized pig was not higher than the levels measured in the other pigs. We also determined the antibody levels in the pigs that were immunized with the plasmid expressing PRRSV-N with the whole viral antigens. However, we did not observe significant increases in the production of antibodies to the whole viral antigens (data not shown). This result confirms that the PRRSV-N protein alone would not harbor the capacity to induce antibody. By way of contrast, the PRRSV-N protein seemed to function properly as a potent immunogenic capacity to induce antibody. By way of contrast, the PRRSV-N protein seemed to function properly as a potent immunogenic capacity to induce antibody.

T-cell proliferation. Autologous porcine PBMCs and DCs were used as APCs to stimulate antigen-specific memory T cells. The PBMCs and DCs were transduced with either TAT-conjugated PRRSV-M or PRRSV-N protein prior to coculture with PBMCs acquired from immunized and nonimmunized pigs. In preliminary experiments, we determined T-cell proliferation at a variety of E/APC ratios, such as 0.5:1 (5 × 10^5:1 × 10^6 cells/ml), 1:1 (1 × 10^5:1 × 10^6 cells/ml), and 5:1 (5 × 10^5:1 × 10^6 cells/ml). The T-cell proliferation responses were noted in the PBMCs obtained from the three pigs immunized with pcDNA3.1-GM-CSF-PRRSV-N (Fig. 7B). The overall T-cell proliferation responses induced by PRRSV-M after 3 days of antigenic stimulation (Fig. 7A). PRRSV-N-specific T-cell proliferation was also noted after 3 days of stimulation in the PBMCs of the pigs immunized with pcDNA3.1-GM-CSF-PRRSV-N. The overall T-cell proliferation responses induced by PRRSV-M after 3 days of antigenic stimulation (Fig. 7A). PRRSV-N-specific T-cell proliferation was also noted after 3 days of stimulation in the PBMCs of the pigs immunized with pcDNA3.1-GM-CSF-PRRSV-N. Three immunized pigs (N#1 to N#3, respectively) and two nonimmunized pigs (contols 1 and 2 [Cont#1 and Cont #2, respectively]) were assessed.

Cytokine production from stimulated T cells. The cytokine production patterns were analyzed in the PBMCs obtained from the immunized and nonimmunized pigs by stimulating them with the autologous APCs transduced with the TAT-PRRSV-M or the TAT-PRRSV-N protein. The concentrations of IFN-γ, IL-6, and IL-10 were determined with cell culture supernatants from unstimulated and stimulated PBMCs. The titers of IFN-γ, IL-6, and IL-10 were determined with cell culture supernatants from unstimulated and stimulated PBMCs.
medium samples obtained from the antigen-stimulated effector cells by ELISA. The T cells in the PBMCs obtained from pigs immunized with pcDNA3.1-GM-CSF-PRRSV-M produced more IFN-γ than the T cells in the PBMCs obtained from pigs immunized with pcDNA3.1-GM-CSF-PRRSV-N after in vitro stimulation with autologous PBMCs expressing PRRSV-M and PRRSV-N, respectively (Fig. 8A and B). The mean concentrations of IFN-γ produced from the two T-cell populations were 605 and 478 pg/ml, respectively. Similar results were noted for the T cells of PBMCs obtained from the pigs immu-
nized with the two DNA vaccines, under conditions in which they were stimulated with DCs expressing the PRRSV-M and PRRSV-N antigens, respectively (Fig. 8C and D). However, in the case of the other cytokines, such as IL-6 and IL-10, their yields did not differ significantly from those generated in the T cells of the nonimmunized pigs. Their levels of IL-6 and IL-10 were consistently below 100 pg/ml (data not shown). These results would appear to indicate that the PRRSV-M protein has a more profound T-cell immunogenic effect than the PRRSV-N protein in immunized pigs.

**DISCUSSION**

In this study, we compared the different immune responses induced in conventional pigs that were systematically immunized with plasmid DNAs encoding the GM-CSF-fused M and N proteins of PRRSV. We confirmed that the M protein has a profound capacity to induce cell-mediated immunity and that this ability of the M protein is significantly greater than that of the N protein. The methodology presented herein represents a practical approach that can be utilized for the primary scanning of other structural or nonstructural proteins of PRRSV, allowing the identification of the components principally involved in cell-mediated immunity. Since GM-CSF has previously been demonstrated to have potent adjuvant activity (33, 34, 45), we fused the porcine GM-CSF gene with the PRRSV-M and PRRSV-N genes in two plasmid DNAs to enhance the immune responses to the M and N proteins in the immunized pigs. We did not address the adjuvant effect of GM-CSF but focused principally on the distinct cellular and humoral immune responses generated by the M and N proteins commonly conjugated with GM-CSF. Despite the intrinsic difficulty presented by DNA immunization, which in our case required a series of repeated immunizations to develop a measurable cell-mediated response, this approach allowed us to compare the immunogenic characteristics of two immunogens. During the preparation of the manuscript, we referred to a recently published report showing that GM-CSF could augment the humoral and cellular immune responses to GP3 and GP5 of PRRSV in vaccinated pigs (40).

We also utilized the intact protein delivery capacity of the HIV-1 TAT gene to generate autologous APCs. The recombinant PRRSV-M and PRRSV-N proteins fused with the HIV-1 TAT gene were successfully translocated into primary porcine cells, such as PBMCs and DCs. When the porcine PBMCs and DCs that were transduced with the TAT-conjugated PRRSV-M or PRRSV-N protein were cocultured with PBMCs, they induced T-cell proliferation. This phenomenon was identified only in pigs immunized with the plasmids expressing PRRSV-M and PRRSV-N and not in the nonimmunized pigs. These results clearly indicate that the memory T cells generated in the pigs immunized with the PRRSV-M and PRRSV-N proteins could recognize the PRRSV-M and PRRSV-N antigens presented by the MHC-matched autologous APCs. Therefore, we anticipate that this newly developed protocol for the generation of MHC-matched autologous APCs should facilitate the analysis of T-cell responses in outbred experimental pigs after infection with various viruses or vaccines.

The principal characteristics of PRRSV infection are persistence and the deterred clearance of virus owing to the delay of IFN-γ and neutralizing antibody production in infected pigs (22, 26, 42). The T cell-mediated immune responses represented by IFN-γ are important in the control of PRRSV infections (1, 23). The study established with the PRRSV-modified live vaccines has also suggested that cell-mediated immune responses are the primary protective mechanism (47). Therefore, it is important to determine which PRRSV proteins are involved in the induction of IFN-γ. In a previously published report, the PRRSV-M protein induced the strongest T-cell responses, whereas the PRRSV-N protein induced a rather weak T cell-stimulating capacity (2). Such features of the PRRSV M and N structural proteins have been confirmed in this study. Under *in vitro* conditions, the stimulation of T cells with the APCs expressing the PRRSV-M antigen always generated significantly higher yields of IFN-γ than those detected with PRRSV-N. On the other hand, it was also suggested that the PRRSV-N protein-mediated cellular immune responses might prove relevant to efforts in the control of viral replication (29, 43). Therefore, more careful evaluations may be required to determine the T cell-mediated immune responses to the PRRSV-N protein. The recent identification of immunodominant T-cell epitopes in the N protein implies that the N protein also performs an important function in cellular immunity to PRRSV (9).

Another feature of immune responses in PRRSV-infected pigs is the increased production of IL-10, a typical suppressor of Th1-type immune responses (8, 35). The increased level of IL-10 expression would be expected to exert a suppressive effect on the expression of IFN-γ and TNF-α, both of which evidence antiviral activities (4). By way of contrast, the reduction of the level of IL-10 production in PRRSV-infected pigs was associated with viral clearance (17). It was also reported that pigs infected *in utero* evidenced enhanced expression of IL-6 and IL-10 mRNAs from their PBMCs (10). Therefore, these two cytokines mediate the suppression of the immune responses necessary for viral clearance in the PRRSV-infected pigs. We measured the levels of production of IL-6 and IL-10 in order to determine which cytokines are induced by the PRRSV-M and PRRSV-N proteins. However, the levels of IL-6 and IL-10 production were rather low in both the immunized and the control pigs. Therefore, the individual PRRSV-M and PRRSV-N proteins do not appear to induce T cells to generate these cytokines compared to the induction ability of whole virus.

Antibodies to the major structural proteins of PRRSV (GP5, M, and N) are generally detected within 1 to 2 weeks postinfection (21, 44). Although antibodies to N protein are the most abundant, they generally do not have neutralizing activity. Instead, antibodies against GP5 function as the most effective neutralizing antibodies (13, 44). In this study, we confirmed that the PRRSV-N protein functions as a good immunogen for the earlier and higher level of induction of antibodies compared to the time of antibody induction and the level of antibody induced by the M protein. The antibodies to the N protein, however, did not neutralize the virus (21, 44). Our laboratory results also confirmed that the N-specific antibody had no neutralizing effect (data not shown). It is known that two major B-cell epitopes are located at the C-terminal region of the PRRSV-M protein (7). In this study, we utilized the
plasmid DNA encompassing the B-cell epitope region of the PRRSV-M gene to immunize the experimental pigs. However, we detected no M protein-specific antibodies in the DNA-immunized pigs. This sort of insufficient or absent antibody production was occasionally detected in the pigs immunized with plasmid DNAs expressing the M protein (19). M and GP5 exist as heterodimers linked by disulfide bonds in the virus particles and infected cells (24). Recombinant vaccinia virus coexpressing the GP5 and M proteins generated more antibodies to the M protein in infected mice. However, no antibodies were detected when the mice were infected only with M protein-expressing vaccinia virus (46). Therefore, we suggest that the M protein expressed by plasmid DNA in our case was not sufficient, in either its function or its conformation, to induce antibody in the pigs without interacting with GP5.

In conclusion, use of the plasmid DNA approach and autologous APCs was shown to be useful for the characterization of the PRRSV structural proteins that induce cell-mediated immunity. The PRRSV-M protein served as a potent antigen for T cell-mediated immune responses but proved insufficient to elicit antibodies by itself. By way of contrast, the PRRSV-N protein generated a high level of antibody and also some degree of T-cell response. The identification of T-cell epitopes on the PRRSV-M protein may provide us with some valuable information and help us to gain a better understanding of immunity to PRRSV.

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