Inclusion of the Bovine Neutrophil Beta-Defensin 3 with Glycoprotein D of Bovine Herpesvirus 1 in a DNA Vaccine Modulates Immune Responses of Mice and Cattle

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Bovine herpesvirus 1 (BoHV-1) causes recurrent respiratory and genital infections in cattle and predisposes them to lethal secondary infections. While modified live and killed BoHV-1 vaccines exist, these are not without problems. Development of an effective DNA vaccine for BoHV-1 has the potential to address these issues. As a strategy to enhance DNA vaccine immunity, a plasmid encoding the bovine neutrophil beta-defensin 3 (BNBD3) as a fusion with truncated glycoprotein D (tgD) and a mix of two plasmids encoding BNBD3 and tgD were tested in mice and cattle. In mice, coadministration of BNBD3 on the separate plasmid enhanced the tgD-induced gamma interferon (IFN-γ) response but not the antibody response. BNBD3 fused to tgD did not affect the antibody levels or the number of IFN-γ-secreting cells but increased the induction of tgD-specific cytotoxic T lymphocytes (CTLs). In cattle, the addition of BNBD3 as a fusion construct also modified the immune response. While the IgG and virus-neutralizing antibody levels were not affected, the number of IFN-γ-secreting cells was increased after BoHV-1 challenge, specifically the CD8+ IFN-γ+ T cells, including CD8+ IFN-γ+ CD25+ CTLs. While reduced virus shedding, rectal temperature, and weight loss were observed, the level of protection was comparable to that observed in pMASIA-tgD-vaccinated animals. These data show that coadministration of BNBD3 with a protective antigen as a fusion in a DNA vaccine strengthened the Th1 bias and increased cell-mediated immune responses but did not enhance protection from BoHV-1 infection.

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used as an experimental model. In large animals, much weaker responses have been observed, which were not always sufficient to provide protective immunity (18). To address this, several methods have been employed to increase the potency of DNA vaccines and/or manipulate the immune response, including improving cellular delivery of plasmid DNA, increasing antigen production, and genetic adjuvanting (23–26).

One method of genetic adjuvanting that has shown promise in the mouse model (27, 28) and also in the chicken (29) to improve (Fig. 1). Complementary oligonucleotide pairs, shown in Table 1, were constructed both in mice and in cattle.

TG D-specific immune response induced by DNA immunization with phosphorylation (BNBD3-2), and the third with a BNBD3 was inserted into pMASIA and pMASIA-tgD (35) in three pieces (Fig. 1). Complementary oligonucleotide pairs, shown in Table 1, were synthesized (Sigma-Aldrich, St. Louis, MO, USA) based on the published coding sequence for the mature BNBD3 peptide (GenBank accession no. AF016396). The first piece was synthesized with a 5' BamHI site (BNBD3-1), the second with 5' phosphorylation (BNBD3-2), and the third with a 3' HindIII site (BNBD3-3). BNBD3-3 was modified for insertion into pMASIA-tgD to create the defensin-viral antigen fusion construct BNBD3-tgD by removing the stop codon and adding the coding sequence for a defined octapeptide linker, NDAQAPKS (36–38), to the 3' carboxy end of the BNBD3-coding sequence to the 5'.

Expression of BNBD3 and BNBD3-tgD in vitro. COS-7 cells at 70% confluence were transiently transfected with pMASIA-BNBD3 or pMASIA-BNBD3-tgD using Lipofectamine Plus reagent in Opti-MEM (Life Technologies). After 48 h, the cell supernatants were collected, clarified by centrifugation, and concentrated 10X using an Amicon Ultra 10kDa (pMASIA-tgD, pMASIA-BNBD3-tgD) or Amicon Ultra 3kDa (pMASIA-BNBD3) centrifugal filter (Millipore, Bedford, MA, USA). Supernatants were divided to provide samples for Coomassie blue staining and Western blotting and were prepared in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer in the presence or absence of 2-mercaptoethanol.

Proteins were separated by SDS-PAGE on a 10% acrylamide gel (pMASIA-tgD and pMASIA-BNBD3-tgD) according to the method of Laemmli (41) or by Tricine SDS-PAGE on a 15% acrylamide gel (pMASIA-BNBD3) according to the method of Shagger et al. (42, 43). Proteins were transferred onto 0.45-μm nitrocellulose (pMASIA-tgD and pMASIA-BNBD3-tgD) or 0.2-μm polyvinylidene difluoride (PVDF) (pMASIA-BNBD3) membranes. The membranes were washed in TBST (0.15 M Tris, 0.02 M NaCl [pH 7.5], 0.1% Tween 20) and then incubated for 2 h at room temperature (RT) or overnight at 4°C in TBST containing 3% skim milk powder (SMP). Membranes were probed with gd-specific monoclonal antibody (Mab) 3D9S diluted 1:4000 in TBST–1% SMP (44) or with BNBD3-specific rabbit serum diluted 1:2000 in TBST–1% SMP for 2 h at RT. Polyclonal antibodies to BNBD3 were generated as described previously (45). The synthetic peptide QGVRNHVTCRINRGFAIPG for 2 h at RT. Polyclonal antibodies to BNBD3 were generated as described previously (45). The synthetic peptide QGVRNHVTCRINRGFAIPG was conjugated to keyhole limpet hemocyanin (KLH) in a molar ratio of 1:1 and used to boost immunity. Mice were boosted with KLH conjugates three times at 2-week intervals and immunized with the peptides in complete Freund’s adjuvant (CFA).

MATERIALS AND METHODS

Construction of plasmids. Expression plasmids encoding the mature form of the CNBD3 peptide (GenBank accession no. AF016396) were constructed according to the strategy shown schematically in Fig. 1. BNBD3 was inserted into pMASIA and pMASIA-tgD (35) in three pieces (Fig. 1). Complementary oligonucleotide pairs, shown in Table 1, were synthesized (Sigma-Aldrich, St. Louis, MO, USA) based on the published coding sequence for the mature BNBD3 peptide (GenBank accession no. AF016396). The first piece was synthesized with a 5' BamHI site (BNBD3-1), the second with 5' phosphorylation (BNBD3-2), and the third with a 3' HindIII site (BNBD3-3). BNBD3-3 was modified for insertion into pMASIA-tgD to create the defensin-viral antigen fusion construct BNBD3-tgD by removing the stop codon and adding the coding sequence for a defined octapeptide linker, NDAQAPKS (36–38), to the 3' carboxy end of the BNBD3-coding sequence to the 5' amino terminus of the sequence for tgD. The coding sequence for this linker has been described previously as 5'-AAC GAC GCA CAA GCA CCA AAA AGC/ TCA-3' (37, 39). Since its use has been primarily in a mouse model, this linker gene was redesigned, to 5'-AAC GAC GCC CAG CCC CCA AAG/ AGC/TCA-3', to optimize the codon bias in favor of expression in bovine cells (http://www.kazusa.or.jp/codon/) (40).

Synthetic oligonucleotides were suspended, and the complementary pairs were annealed according to the supplier's instructions. The three double-stranded sticky-ended oligonucleotide pieces encoding BNBD3 were then ligated into the BamHI/HindIII sites of pMASIA and pMASIA-tgD, respectively. The ligation reactions were used to transform Escherichia coli strain JM109, and after selection (Kan+ and ampicillin), the plasmids were purified using a Qiagen Miniprep kit (Qiagen). Correctness of pMASIA-BNBD3 and pMASIA-BNBD3-tgD was verified by restriction digestion and confirmed by DNA sequencing. Plasmids were amplified in Escherichia coli JM109 cells and purified with Endoforce Plasmid Giga kits (Qiagen).

Expression of BNBD3 and BNBD3-tgD in vivo. Six- to 8-week-old C56BL/6 mice (8 mice per group) were immunized twice at a 4-week interval intradermally (i.d.) at the base of the tail with 5 μg pMASIA, pMASIA-tgD, pMASIA-BNBD3-tgD, or a mixture of pMASIA and pMASIA-tgD or pMASIA-BNBD3 and pMASIA-tgD. One month after the final vaccination, mice were euthanized. Serum was taken to be assayed for TG D-specific antibody levels, and spleens were collected for analysis of tgD-specific cell-mediated immune (CMI) responses.

Eight- to 9-month-old BoHV-1-seronegative Angus and Hereford crossbred calves were randomly allocated to five groups of six animals each and immunized with 750 μg pMASIA, pMASIA-tgD, pMASIA-BNBD3-tgD, or a mixture of pMASIA and pMASIA-tgD, or a mixture of pMASIA-BNBD3 and pMASIA-tgD. The plasmids were delivered three times at 4-week intervals i.d. in the neck with a needle-free delivery device (Biojector Medical Technologies, Portland, OR). One month after each vaccination, serum was collected for analysis of TG D-specific antibody levels and peripheral blood was collected for analysis of CMI responses.

Calves in the groups immunized with pMASIA, pMASIA-tgD, or pMASIA-BNBD3-tgD were challenged intranasally with 4 ml of aerosol-
ized BoHV-1 strain 108 (10^7 PFU/ml) at 52 days after the last immunization. Sera were collected prior to and on days 8 and 16 after challenge. Peripheral blood was collected and CMI responses were measured prior to challenge and on day 8 after challenge by proliferation and enzyme-linked immunosorbent spot (ELISPOT) assays, on day 16 by ELISPOT depletion assay, and on day 25 by a 4-color CTL assay. Calves were clinically assessed before challenge and for 10 days after challenge. Temperatures and body weights were measured and nasal swabs were collected every second day. All procedures were approved by the University Council for Animal Care and Supply in accordance with the standards stipulated by the Canadian Council on Animal Care.

**Serology.** For the enzyme-linked immunosorbent assay (ELISA), 96-well polystyrene microtiter plates (Immulon 2; Thermo Electron Corp., Milford, MA) were coated overnight at 4°C with 0.05 μg of tgD per well in sodium carbonate coating buffer. Plates were washed in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST) and then incubated overnight at 4°C with serially diluted mouse sera starting at 1:40 in 4-fold dilution, with all dilutions in PBS containing 0.5% gelatin (PBS-g). Plates were washed, and bound IgG was detected using AP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) diluted 1:5,000 in PBS-g for 1 h at room temperature (RT). Bovine sera were diluted 4-fold in PBS-g starting at 1:10, added to plates, and incubated for 2 h at RT. The plates were then incubated for 1 h at RT with affinity-purified AP-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratories) diluted 1:10,000 in PBS-g. All reactions were visualized with 0.01 M p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich) in 0.104 M diethanolamine–0.5 mM MgCl. Absorbance was read on a model 3550 microplate reader (Bio-Rad Laboratories Ltd.) at 405 nm, with a reference wavelength of 490 nm.

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**FIG 1** Schematic diagram of the construction of pMASIA-tgD, pMASIA-BNBD3, and pMASIA-BNBD3-tgD. The gene encoding BoHV-1 tgD was cloned into pMASIA to create pMASIA-tgD. To construct pMASIA-BNBD3, complementary oligonucleotides encoding the mature sequence of BNBD3 were synthesized as 3 sticky-ended pieces (BNBD-1, BNBD-2, and BNBD-3) and inserted into pMASIA using the BamHI and HindIII sites. For construction of pMASIA-BNBD3-tgD, BNBD3 was similarly inserted in 3 pieces into pMASIA-tgD, and only the third piece (BNBD3-3L) differed, with a linker sequence at the 3' end.
TABLE 1 Primers used for PCR amplification and BNBD3-encoding complementary synthetic oligonucleotide pairs

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer or oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNBD3-1</td>
<td>5′-GATCCTATGCGAAGGATTGAATACGGATGACGTCGATTGTCGACGTCGATCAT-3′</td>
</tr>
<tr>
<td>BNBD3-1c</td>
<td>5′-CAGAAGGCTCTATTTATACGCCGATACATGTTTCTTACTCTGCGATATG-3′</td>
</tr>
<tr>
<td>BNBD3-2</td>
<td>5′-TCCTGTGGCGATGCGTCGATCGGCCGAGCAAGCGACAGACCAAGATGACGCCATGGTCGACGTCGATCATG-3′</td>
</tr>
<tr>
<td>BNBD3-2c</td>
<td>5′-GCCGAGAACGCTGACATGCTCTGCGTCGATCAAGGCGCTGAGCGGACAC-3′</td>
</tr>
<tr>
<td>BNBD3nL-3</td>
<td>5′-GGGCGGGCAATAATAGTCGAGTGCTCGGAGACGACGGGACCGAGCCGACGACGAACATGACGCCATGGTCGACGTCGATCATG-3′</td>
</tr>
<tr>
<td>BNBD3nL-3c</td>
<td>5′-AGCCTCTACACGCGCTGAGCATTTATATTTCGACGCGCCGAGCGGACACGACGGGACCGACGACGAACATGACGCCATGGTCGACGTCGATCATG-3′</td>
</tr>
<tr>
<td>BNBD3L-3</td>
<td>5′-GGGCGGGCAATAATAGTCGAGTGCTCGGAGACGACGGGACCGAGCCGACGACGAACATGACGCCATGGTCGACGTCGATCATG-3′</td>
</tr>
<tr>
<td>BNBD3L-3c</td>
<td>5′-AGCCTCTACACGCGCTGAGCATTTATATTTCGACGCGCCGAGCGGACACGACGGGACCGACGACGAACATGACGCCATGGTCGACGTCGATCATG-3′</td>
</tr>
</tbody>
</table>

*First piece of BNBD3; a 5′ BamHI restriction site is underlined, and the start codon is in bold.

*Second piece of BNBD3; oligonucleotides were 5′ phosphorylated, and sticky ends were made by a 4-nucleotide overhang (italic).

*Third piece of BNBD3 (pMASIA-BNBD3); a 3′ HindIII restriction site is underlined, and the TAG stop codon is in bold.

*Fourth piece of BNBD3 (pMASIA-BNBD3-tgD); the bovine codon optimized linker is in italic, and a 3′ HindIII restriction site is underlined.

dFirst piece of BNBD3; a 5′ BamHI restriction site is underlined, and the start codon is in bold.

dSecond piece of BNBD3; oligonucleotides were 5′ phosphorylated, and sticky ends were made by a 4-nucleotide overhang (italic).

dThird piece of BNBD3 (pMASIA-BNBD3-tgD); the bovine codon optimized linker is in italic, and a 3′ HindIII restriction site is underlined.

ELISA titers were expressed as the inverse of the serum dilution that gave an absorbance (A) value two standard deviations above the values for sera from control naïve animals.

Virus neutralization (VN) titers in cattle serum were determined as described previously (48). Viral plaques were visualized by staining each well with 20 μl of 0.5% crystal violet in 80% methanol for 1 min, and then counted. Titers were expressed as the reciprocal of the highest dilution of serum that resulted in a 50% reduction in plaques relative to the serum-control group. Titers were expressed as the reciprocal of the highest dilution of serum that resulted in a 50% reduction in plaques relative to the serum-control group. For cattle, the enzyme-linked immunosorbent spot (ELISPOT) assay

**Samples were made by a 4-nucleotide overhang (italic).**

**Proliferation:** [methyl-3H]thymidine incorporation assay. Bovine blood was collected, and PBMCs were isolated and suspended as described above. Cells were further diluted to a concentration of 0.5 x 10^6 cells/ml in cRPMI. One hundred microliters of this suspension was dispensed in triplicate into wells containing 100 μl of either medium or tgD at 3 μg/ml, and then plates were incubated at 37°C. Following 72 h of restimulation, the cells were pulsed with 0.4 μCi/well of [methyl-3H]thymidine (Amersham Biosciences, Baie d’Urfe, PQ, Canada). After an additional 18 h of culture, cells were collected with a Filtermate harvester, and thymidine uptake was measured by scintillation counting with a TopCount NXT microplate scintillation counter (Packard Instrument Company, Meriden, CT, USA). Proliferative responses were calculated as the means from triplicate wells and expressed as a stimulation index (SI) (counts per minute in the presence of antigen/counts per minute in the absence of antigen).

**Antigen-specific CD8** + IFN-γ cytoxic T cell assay. In mice the induction of CTLs, defined as CD8 + CD3 + T cells that when stimulated by antigen secretes IFN-γ (52–55), was measured by fluorescence-activated cell sorting (FACS). Splenocytes were isolated from each mouse group and resuspended as described above. Pooled splenocytes (55, 56) from each vaccination group were added to wells of a 96-well plate at 1 x 10^6 cells/well and then cultured at 37°C in the absence of the presence of tgD at 3 μg/ml for 7 h. GolgiPlug (BD Biosciences) was added at 5 h before harvesting the cells. Cells were washed once with FACS buffer (PBS [pH 7.2], 0.1% BSA, 0.05% NaNO3) supplemented with 2% FBS and cell surface stained with phycoerythrin (PE)–anti-mouse CD3 (IgG2b) (clone 145-2C11-2; BD Biosciences) and fluorescein isothiocyanate (FITC)–anti-mouse CD8 (clone 53-6.7; BD Biosciences) at 4°C for 30 min. Intracellular cytokine staining of IFN-γ–secreting cells was performed, fixed, and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Biosciences). Intracellular IFN-γ was stained with allophycocyanin (APC)–anti-mouse IFN-γ (IgG1) (clone XMG1.2; BD Biosciences).

In cattle, due to difficulty doing CTL assays in outbred animals, the induction of activated CD8 + IFN-γ + cells in the PBMCs was used as a measurement of CTL response using a FACS-based assay. This assay has been accepted as an alternative in other species to the standard cytolytic (Cr51 release) assay (57, 58) and was also selected based on a published report of CTL identification in cattle as activated, CD8 + IFN-γ–expressing cells (59). Accordingly, CD8 + IFN-γ + expression, concurrent with CD25 activation, was used as a surrogate marker for CTLs (53, 60), and thus cells were identified using a modification of a method that allows for simultaneous detection of expression of CD8 + and IFN-γ + with the cell activation marker CD25 (61–64). Optimal conditions for cell collection and culture were determined in preliminary studies. Blood from two animals from the pMASIA-tgD, pMASIA-BNBD3-tgD, and pMASIA groups was collected into heparin. PBMCs were isolated on Ficoll-Paque Plus (Pharmacia) (32), suspended in cRPMI, and then added at 1 x 10^6 cells per well in a 250-μl volume of a 96-well round-bottom tissue-culture microtiter plate (Corning Costar; Thermo Fisher Scientific, Life Technol-
cattle (53, 56, 59, 60, 65) in response to

2, synthesized BNBD3 (sBNBD3) peptide (1 μg); 3, sBNBD3 peptide (0.5 μg); 4, pMASIA supernatant; 5, pMASIA-BNBD3 supernatant. (b and d) Lanes: 1, molecular mass marker; 2, pMASIA-tgD supernatant; 3, pMASIA-BNBD3-tgD supernatant; 4, pMASIA-BNBD3-tgD supernatant. (c) Lanes: 1, molecular mass marker; 2, pMASIA-tgD supernatant; 3, pMASIA-BNBD3 supernatant. (d) Lanes: 1, molecular mass marker; 2, pMASIA supernatant; 3, pMASIA-BNBD3 supernatant; 4, pMASIA-BNBD3-tgD supernatant.

FIG 2 In vitro expression of the BNBD3-encoding constructs. COS-7 cells were transiently transfected with pMASIA-BNBD3 (a and c) or pMASIA-BNBD3-tgD (b and d). The presence of BNBD3 and tgD in the supernatants of transfected cells was detected at 48 h posttransfection by Western blotting with rabbit anti-BNBD3 polyclonal or anti-gD monomeric antibodies under reducing (a and b) and nonreducing (c and d) conditions. (a and c) Lanes: 1, molecular mass marker; 2, synthesized BNBD3 (sBNBD3) peptide (1 μg); 3, sBNBD3 peptide (0.5 μg); 4, pMASIA supernatant; 5, pMASIA-BNBD3 supernatant. (b and d) Lanes: 1, molecular mass marker; 2, pMASIA supernatant; 3, pMASIA-tgD supernatant; 4, pMASIA-BNBD3-tgD supernatant.

ogies Inc., Burlington, ON, Canada). The cells were cultured at 37°C in 2 ml of prediluted streptavidin-Alexa Fluor 647 conjugate (streptavidin S-32357; Molecular Probes, Invitrogen) was added and left for 30 min at 4°C. After washing twice with FAC buffer and surface stained using an indirect staining method. Cells were incubated singly with mouse anti-bovine CD8 MAB (IgG1, MM1; VMRD, Pullman, WA, USA) and singly or with a MAb mix that consisted of (i) mouse anti-bovine CD8 (IgG1, CACTM0C, VMRD), mouse anti-bovine gamma-delta T cell receptor (y6TCR) (IgG2b, GB21A, VMRD), and mouse anti-bovine CD25 (IgG2a, CACT108A, VMRD) or (ii) mouse anti-bovine CD4 (IgG1, CACT138A, VMRD), mouse anti-bovine y6TCR, and mouse anti-bovine CD25 for 30 min at 4°C. After washing twice with FACs buffer, cells were incubated for 30 min at 4°C either singly or with a mixture of the appropriate secondary reagent(s) goat F(ab’2) anti-mouse IgG1–FITC (1072-02; Southern Biotech, Birmingham, AL, USA), goat anti-mouse IgG2b–Tri-Color (M32406; Molecular Probes, Life Technologies Inc.), and goat F(ab’2) anti-mouse IgG2a–R-PE (1082-09; Southern Biotech). Intracellular cytokine staining of IFN-γ was accomplished with a two-step indirect method using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions with the following exceptions. Cells were fixed and permeabilized by incubating at 4°C for 1.5 h. Cells were washed twice and resuspended in 1× BD Perm/Wash for both steps. In the first step, 25 μl of prediluted biotinylated mouse anti-bovine IFN-γ (biotin MCA1783B; AbD Serotec) was added and left for 1 h at 4°C. In the second step, 25 μl of prediluted streptavidin-Alexa Fluor 647 conjugate (streptavidin S-32357; Molecular Probes, Invitrogen) was added and left for 30 min at 4°C.

Staining specificity was controlled with the appropriate isotype-matched antibody controls. Samples were resuspended in flow buffer (PBS, 1% ultrapure formaldehyde) and kept in the dark at 4°C until flow cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multicolor-stained samples. Cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multicolor-stained samples. Cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multicolor-stained samples. Cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multicolor-stained samples. Cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multicolor-stained samples.

Statistical analysis. All data were analyzed with the aid of GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). For mouse and cattle prechallenge ELISA titers and ELISPOT counts, cattle prechallenge proliferation assay SIs, and cattle postchallenge ELISPOT counts, differences among groups were examined using the nonparametric Kruskal-Wallis test. If the result of an analysis of variance (ANOVA) proved significant, then multiple posttest comparisons between medians were done using Dunn’s test, or differences between the medians of two groups were examined using the Mann-Whitney U test. Postchallenge cattle ELISA and virus neutralization titer, virus shedding, weight change, and temperature differences among groups over time were analyzed by a two-way ANOVA followed by a Bonferroni t test in case of a significant ANOVA result. Differences between groups were considered significant if P values of <0.05 were obtained.

RESULTS

In vitro expression of BNBD3 and BNBD3-tgD in COS-7 cells. Expression of BNBD3 from pMASIA-BNBD3 was verified by Western blotting. A single band of the expected molecular mass (4.8 kDa) (46) that comigrated with the synthesized BNBD3 control (Fig. 2a and c, lanes 2 and 3) was detected in the supernatants from pMASIA-BNBD3-transfected cells (Fig. 2a and c, lanes 5) under both reducing (Fig. 2a) and nonreducing (Fig. 2c) conditions. However, BNBD3 was not detected in the supernatants from pMASIA-tgD-transfected cells (Fig. 2a and c, lane 4). A protein corresponding to the expected molecular mass of tgD (61 kDa), was revealed by a gD-specific MAB in supernatants from transfections with pMASIA-tgD (Fig. 2b and d, lanes 3, right panels) or pMASIA-BNBD3-tgD (Fig. 2b and d, lanes 4, right panels) under both reducing (Fig. 2b) and nonreducing (Fig. 2d) conditions, thus verifying expression of tgD from both plasmids. The BNBD3-specific rabbit serum reacted only with the supernatant from pMASIA-BNBD3-tgD transfection (Fig. 2b and d, lanes 4, left panels) and not with the supernatant from pMASIA-tgD transfection (Fig. 2b and d, lanes 3, left panels) under both reducing (Fig. 2b) and nonreducing (Fig. 2d) conditions. As expected, there was little difference in the molecular mass of the fusion protein BNBD3-tgD, which is approximated as 65.4 kDa, and that of tgD (61 kDa) due to the small molecular mass of BNBD3 (4.8 kDa). These results verified the expression of BNBD3 and BNBD3-tgD,
both in monomeric form, from eukaryotic cells transfected with pMASIA-BNBD3 and pMASIA-BNBD3-tgD, respectively.

Immune responses induced by BNBD3-encoding DNA vaccines in mice. The capacity of BNBD3, encoded either on a separate plasmid or as a fusion construct with tgD, to enhance tgD-specific immune responses was first evaluated in mice. Mice immunized with pMASIA plus pMASIA-tgD or pMASIA-BNBD3 plus pMASIA-tgD developed significantly higher IgG titers than the animals immunized with pMASIA (P < 0.01), but there was no difference between these groups (Fig. 3a). The IgG titer of the group immunized with pMASIA-BNBD3-tgD appeared to be lower than, but was not significantly different from, that of the pMASIA-tgD group. However, only mice immunized with pMASIA-tgD, and not those immunized with pMASIA-BNBD3-tgD, developed significantly higher IgG titers than the group immunized with pMASIA (P < 0.05) (Fig. 3a). These results demonstrate that despite these trends, BNBD3 had no significant effects on the humoral immune response induced by tgD.

To examine the effects of BNBD3 on the CMI responses, tgD-induced secretion of IFN-γ and IL-5 by splenocytes was measured by ELISPOT assay. Mice immunized with pMASIA-BNBD3 plus pMASIA-tgD had a significantly higher number (P < 0.001) of IFN-γ-secreting cells upon restimulation with tgD than the animals immunized with pMASIA and than their control group immunized with pMASIA plus pMASIA-tgD (P < 0.05) (Fig. 3b). Immunization with pMASIA-BNBD3-tgD increased the number of IFN-γ-secreting cells (P < 0.01) compared with pMASIA, but there was no significant difference with pMASIA-tgD. The number of cells expressing IL-5 was very low in all vaccinated groups (Fig. 3c). These results indicate that addition of BNBD3 by a separate plasmid resulted in increased numbers of IFN-γ-secreting splenocytes, while BNBD3 added to tgD as a fusion had no effect on the IFN-γ response in mice.

In earlier DNA vaccine studies involving mBD2 in the mouse model, it was found that physical linkage between the beta-defensin and antigen was required for improvement of immune responses (27, 28, 36) and that this may have been particularly important for development of the CTL response (28). To investigate the influence of BNBD3 as a fusion with tgD on the induction of CD8+ IFN-γ+ CTLs, the tgD-restimulated splenocytes from the mice immunized with pMASIA-BNBD3-tgD were evaluated by FACS analysis for surface expression of CD3 and CD8 and concurrent intracellular expression of IFN-γ. First, the live (single) cells were gated according to forward scatter (FSC) and side scatter (SSC) properties (Fig. 4a) (66). Analysis of the total live cells showed that vaccination with pMASIA-BNBD3-tgD induced a greater percentage of tgD-specific CD8+ IFN-γ+ cells (8.7%) (Fig. 4c and d) than was induced in the group vaccinated with pMASIA-tgD (0.1%) (Fig. 4b and d) or vaccinated with pMASIA (0%) (Fig. 4d). Next, the live cells were further gated on surface expression of CD3 to exclude all non-T cells (Fig. 4e). Analysis of this CD3+ population showed that vaccination with pMASIA-BNBD3-tgD again induced a greater percentage of CD8+ IFN-γ+ cells (16.1%) (Fig. 4g and h) than was induced by vaccination with pMASIA-tgD (11.7%) (Fig. 4f and h) or with pMASIA (0%) (Fig. 4h), but the effect of BNBD3 was not as pronounced in this CD3+ T cell-only population. When the frequencies of CD8+ IFN-γ+ cells in the total live cell population (Fig. 4d) and in the CD3+ cell populations (Fig. 4h) were compared, it appeared that vaccination with pMASIA-BNBD3-tgD induced a population of non-T

FIG 3 tgD-specific immune responses in mice immunized with plasmids encoding tgD and/or BNBD3. C57BL/6 mice (8 mice per group) were immunized twice i.d. with 5 μg plasmid. (a) One month after the second immunization, IgG titers were determined by ELISA. IgG titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. (b and c) One month after the second immunization, the numbers of tgD-specific IFN-γ-secreting cells (b) or IL-5-secreting cells (c) were measured by ELISPOT assay. ELISPOT assay results are expressed as the difference between the number of IFN-γ- or IL-5-secreting cells in tgD-stimulated wells and medium control wells per 10^6 cells. Bars represent the median values for each group with interquartile range. Significant differences between groups are indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Similar results were obtained in a second independent experiment.
cell (CD3⁻) IFN-γ⁺-secreting CD8⁺ cells that vaccination with pMASIA-tgD did not. Since dendritic cells (DCs) are currently the only known non-T CD8⁺ cells in the mouse spleen, it is possible that the CD3⁻ CD8⁺ IFN-γ⁺ cells that we observed were splenic DCs (67). These results demonstrate that addition of BNBD3 as a fusion construct led to enhanced induction of tgD-specific CTLs and thus a Th1-type response.

Immune responses induced by BNBD3-encoding DNA vaccines in cattle. Once it was confirmed that plasmids encoding BNBD3 induced immune responses in mice, we next assessed the effects of these plasmids in cattle. Calves immunized with either pMASIA-BNBD3-tgD or pMASIA-BNBD3 plus pMASIA-tgD did not develop higher IgG titers than animals immunized with pMASIA, whereas calves immunized with pMASIA-tgD or pMASIA plus pMASIA-tgD (P < 0.01) did have a significantly higher IgG titer (Fig. 5c). These results demonstrate that addition of BNBD3, encoded either on a separate plasmid or as a fusion construct with tgD, did not enhance the humoral immune response induced by pMASIA-tgD in cattle.

Proliferative responses of tgD-restimulated PBMCs from calves immunized with pMASIA-BNBD3 plus pMASIA-tgD were not significantly improved compared to those of its control group pMASIA plus pMASIA-tgD, and most importantly, neither group was able to develop significantly higher proliferation than calves in the group immunized with pMASIA (Fig. 5b). In contrast, addition of BNBD3 to tgD as a fusion construct greatly improved the proliferative response compared to that for pMASIA-tgD (P < 0.01). Notably, the group immunized with pMASIA-BNBD3-tgD was the only one with significantly higher proliferation than the group immunized with pMASIA (Fig. 5b). To further confirm activation and to characterize the type of immune response generated, production of IFN-γ was assessed (Fig. 5c). Neither of the two-plasmid immunization strategies, pMASIA plus pMASIA-tgD or pMASIA-BNBD3 plus pMASIA-tgD, induced a significantly greater number of IFN-γ-secreting cells than pMASIA. In contrast, addition of BNBD3 to tgD as a fusion construct resulted in an increase in the number of IFN-γ-secreting cells. Although this increase was not statistically significant compared to its control group pMASIA plus pMASIA-tgD, the significance of the difference observed between pMASIA and pMASIA-BNBD3 plus pMASIA-tgD (P < 0.01) was greater than that of the difference between pMASIA and pMASIA-tgD (P < 0.5). Thus, in cattle the addition of BNBD3 as
a fusion construct, but not when delivered by separate plasmid, enhanced the proliferative response to pMASIA-tgD.

Immune responses of cattle after immunization with DNA vaccines and BoHV-1 challenge. Since BNBD3, when delivered on a separate plasmid, did not improve immune responses to tgD, the groups immunized with pMASIA-BNBD3 plus pMASIA-tgD and pMASIA-tgD were not further evaluated, while the groups immunized with pMASIA, pMASIA-tgD, or pMASIA-BNBD3-tgD were subsequently challenged with BoHV-1. Throughout the period after challenge, calves immunized with pMASIA-tgD (P < 0.01) had higher serum IgG titers than calves immunized with pMASIA (Fig. 6a), while the VN titers were significantly higher in both the pMASIA-tgD- and pMASIA-tgD-BNBD3-immunized groups than in the pMASIA group (Fig. 6b). With exception of the IgG titers on day 16 postchallenge, no differences were observed between the pMASIA-tgD and pMASIA-tgD-BNBD3 groups.
In contrast, addition of BNBD3 to tgD as a fusion construct resulted in a significant increase in the number of IFN-γ-secreting cells (P < 0.05) compared to the pMASIA-tgD group (Fig. 6c). To further characterize the effect of BNBD3 on the cellular immune response, an ELISPOT depletion assay was employed whereby the T cell subsets responsible for secreting IFN-γ were identified by isolating the CD4⁺, CD4⁻, CD8⁺, and CD8⁻ T cell populations from the PBMCs of two calves from each of the groups vaccinated with pMASIA-tgD, pMASIA-BNBD3-tgD, or pMASIA. Typical proportions of the T cell subsets in PBMCs were 24% CD4⁺ cells and 15% CD8⁺ cells (Fig. 7a and d). The purities of the isolated populations were 98% for CD4⁺ and CD8⁺ (Fig. 7b and e) and >90% for CD4⁺ and CD8⁻ (Fig. 7c and f). Notably, we observed 5-fold-higher numbers of IFN-γ-secreting cells in the PBMCs from the calves vaccinated with pMASIA-BNBD3-tgD (Fig. 7i and j) than in those from calves vaccinated with pMASIA-tgD (Fig. 7g and h), suggesting that BNBD3 had a positive influence on the magnitude of the IFN-γ response. The CD4⁺ T cell-depleted PBMCs from calves in the pMASIA-tgD (Fig. 7g) or pMASIA-BNBD3-tgD (Fig. 7i) group did not secrete IFN-γ in response to restimulation with tgD. For both groups, when 5, 10, 15, or 20% CD4⁺ T cells were added to the CD4⁻ T cell-depleted PBMCs, IFN-γ secretion increased dose dependently to an amount equal to that of the undepleted PBMCs (Fig. 7g and i). Depletion of CD8⁺ T cells did not affect the number of IFN-γ-secreting cells in the PBMCs from animals in the pMASIA-tgD group, and there was also no change when 2.5, 5, 10, or 15% CD8⁺ T cells were added to the CD8⁻ T cell-depleted PBMCs (Fig. 7h). In contrast, when PBMCs from the pMASIA-BNBD3-tgD group were depleted of CD8⁺ T cells, a 2-fold reduction in the number of IFN-γ-secreting cells was observed, and there was a dose-dependent increase when 2.5, 5, 10, or 15% CD8⁺ T cells were added to the CD8⁻ T cell-depleted PBMCs (Fig. 7j). These depletion studies showed that CD4⁺ T cells in the PBMCs of calves from both vaccinated groups produced IFN-γ but that only calves in the pMASIA-BNBD3-tgD group produced IFN-γ-secreting CD8⁻ T cells. Thus, the addition of BNBD3 to tgD as a fusion construct appeared to increase the magnitude of the IFN-γ response and to induce tgD-specific CD8⁻ T cells.

To determine whether these CD8⁻ T cells might be CTLs, the PBMCs from the same calves were restimulated with tgD and then evaluated by flow cytometry. Using a multicolor flow cytometry assay, cells were identified as CTLs that, upon restimulation with the recall antigen tgD, simultaneously secreted IFN-γ and expressed CD8 and the alpha subunit of the high-affinity interleukin-2 receptor (IL-2Rα) on the cell surface (also known as the cell activation marker CD25) (61–64). Within the CD8⁻ T cell population, the percentage of cells positive for IFN-γ secretion was almost 2-fold higher, at 36.5% and 36.4%, after challenge in the calves vaccinated with pMASIA-BNBD3-tgD than the 19.7% and 16.3% positive cells observed for calves vaccinated with pMASIA-tgD (Table 2). This increased IFN-γ secretion by CD8⁺ T cells correlated well with, and may account for some of the higher numbers of IFN-γ-producing cells observed in the ELISPOT results (Fig. 6c and 7i and j) from calves in the pMASIA-BNBD3-tgD group. To identify CTLs within this population, coexpression of the CD25 activation marker by these CD8⁺ IFN-γ⁺ T cells was examined. The percentage of CD8⁺ IFN-γ⁺ CD25⁻ cells from calves vaccinated with pMASIA-BNBD3-tgD was 22.1% and 22.7%, representing a greater-than-2-fold increase compared to the 12.5% and 6.9% of CD8⁺ IFN-γ⁺ CD25⁺ cells observed for calves vaccinated with pMASIA-tgD (Table 2). Thus, in good agreement with the results in mice, in cattle the addition of BNBD3 as a fusion construct with tgD increased the proportion of what are considered CTLs, namely, activated, tgD-specific CD8⁻ T cells. No effect of vaccination with the BNBD3-encoding vaccine on the numbers of CD8⁺ IFN-γ⁺ CD25⁺ T cells was observed.

These results demonstrate that in vaccinated, BoHV-1-challenged cattle, the addition of BNBD3 as a fusion construct increased the number of IFN-γ-secreting cells, specifically the number of IFN-γ-secreting tgD-specific CD8⁻ T cells, and notably the CD8⁺ IFN-γ⁺ CD25⁻ subset.

Clinical observations of protection after BoHV-1 challenge. Calves in all three groups shed virus from day 2 after challenge, with significantly less virus shed on day 8 by calves in the pMASIA-BNBD3-tgD (P < 0.01) and pMASIA-tgD (P < 0.05) groups than by those in the pMASIA group (Fig. 8a). By day 10, both the pMASIA-BNBD3-tgD and pMASIA-tgD groups showed equal and significantly less viral shedding (P < 0.05) compared to the pMASIA group (Fig. 8a). Weight loss was observed in calves from all three groups by day 2 to day 4 after challenge (Fig. 8b). On day 6, weight loss was reversed for both the pMASIA-BNBD3-tgD and pMASIA-tgD groups, whereas calves in the pMASIA group continued to lose weight. Additionally, on day 6 only calves in the pMASIA-BNBD3-tgD group (P < 0.05) group had significantly less weight loss than the pMASIA group, while by day 10 calves in both the pMASIA-BNBD3-tgD and pMASIA-tgD groups had equal and significantly less weight loss (P < 0.05) than calves in the pMASIA group (Fig. 8b). Rectal temperatures peaked for all three groups on day 2 after challenge and then began falling (Fig. 8c). Between days 8 and 10, temperatures rose in the pMASIA group, while they fell in the pMASIA-BNBD3-tgD and pMASIA-tgD groups. By day 10 after challenge, rectal temperatures were significantly lower in calves from the pMASIA-BNBD3-tgD group (P < 0.05) and pMASIA-tgD (P < 0.01) group than in those from the pMASIA group (Fig. 8c). These results demonstrate that the addition of BNBD3 as a fusion construct was able to protect cattle from infection with BoHV-1 equally as well as, but not better than, the control DNA vaccine, pMASIA-tgD.

DISCUSSION

In this study, the effect of DNA vaccines encoding an immature DC (iDC)-chcmotactic peptide BNBD3, separately or as a fusion construct with BoHV-1 tgD, on the immune responses was determined in mice and cattle. In mice, addition of pMASIA-BNBD3 to pMASIA-tgD had no effect on tgD-specific IgG and increased the number of IFN-γ-secreting cells, while in cattle this two-plasmid treatment did not improve either humoral or cellular immune responses. When BNBD3 was delivered with tgD as a fusion construct in pMASIA-BNBD3-tgD, again there was no significant effect on tgD-specific antibody production; however, the CMR responses were enhanced in both mice and cattle. The addition of BNBD3 as a fusion construct with tgD induced a greater number of IFN-γ-secreting CD8⁻ T cells. In mice, this construct induced increased numbers of CD8⁺, CD8⁻ T cells and, interestingly, also a population of cells identified as non-T (CD3⁻) IFN-γ⁺ CD8⁻ cells that may have been CD8α⁻ splenic DCs. In cattle, CD8⁺ IFN-γ⁺ cells were increased and CD8⁺ IFN-γ⁺ CD25⁻ CTLs were induced only in animals immunized with pMASIA-BNBD3-tgD. However, despite the fact that immunization with pMASIA-BNBD3-tgD en-
hanced the CMI response, protection from challenge was similar to that provided by immunization with pMASIA-tgD.

Like our results in cattle, in earlier studies in mice, mixtures of free, unlinked murine chemokines and antigen (36) or a mixture of plasmids expressing unlinked antigen and murine beta-defensin (27) did not induc an immune response (27, 28, 36). In our study, segregation of BNBD3 and tgD either physically or temporally may have occurred in cattle and not in mice; this phenomenon of separation has been suggested previously to account for little or no effect when plasmids are mixed (68). Additionally, it has been noted that responses to mixtures of plasmids can result in interference, leading to dominant Th2 responses, less appropriate responses (68), or suppression of responses (69). The outbred nature of cattle as a species might have also contributed to the lack of effect of BNBD3 in cattle when delivered as a separate plasmid, as immune responses to mixtures of plasmids have been found to be lower in outbred mice than in inbred mice (70).

Inclusion of BNBD3 in the DNA vaccine as a fusion construct (pMASIA-BNBD3-tgD) did not affect humoral responses to tgD but increased CMI responses and appeared to strengthen the Th1 bias in mice. Similarly, in cattle this vaccine was unable to improve serum antibody levels, but it increased proliferation of PBMCs and the number of IFN-γ-secreting cells. Our results are comparable with the findings of an earlier study in mice, where the ability of an analogous fusion construct comprised of mBD2 (and mBD3) in combination with idiotypic antigen (Id) expressed by malignant B cells to induce protective and therapeutic immunity to lymphoma was tested (27). sFv, a single-chain Ig made up of the linked Vh and VL domains of the Fv fragment of the Ig receptor of the malignant B cell (described in reference 36) failed to elicit an Id-specific antibody response when the sFv-encoding DNA vaccine was delivered alone or with mBD3 on a separate plasmid, whereas responses were observed after vaccination with fusion constructs of either mBD2, mBD3, mMIP3α, or mSLC (27). Protective immunity against an aggressive lymphoma (38C13) was obtained after DNA vaccination by both mBD-sFv fusion plasmids even though the humoral response was considerably lower with the vaccine encoding mBD2. The authors concluded that fusion of tumor antigen with a chemokine or defensin that targets iDCs was important for both tumor prevention and eradication. While humoral immunity contributed to protection from tumors, cellular antitumor immunity was necessary for both protection and therapeutic antitumor immunity (27). In contrast to our results, this study suggests that the increased CMI responses induced by the defensin-antigen fusion were critical to antitumor efficacy.

In mice the fusion construct pMASIA-BNBD3-tgD modulated the cellular immune response by inducing CD8+ IFN-γ+ CD3+ CTLs and a population of cells that were CD8+ IFN-γ− but that were not T cells. It is possible that these CD8+ IFN-γ− CD3+ cells may have been DCs, since splenic CD8α+ DCs have been described. This type of DC lacks expression of CD3 (67) and is a potent secretor of IFN-γ, where IFN-γ is produced in an autocrine manner in response to IL-12 secreted by the cells exposed to a bacterial stimulus (71). These DCs are expanded in response to signals from the innate immune system as a result of bacterial or viral infection (71–73). They cross-prime (74), prime (73), or prime and boost CD8+ T cell responses and activate memory CD8+ T cells (75), trigger the development of Th1-type cells/response (76, 77), and cause apoptotic death of activated CD4+ T cells (78). As defenses are innate immune system molecules, BNBD3 might have induced or modified CD8α+ DCs in the same manner, as they are increased by bacterial/viral infection, though this was not proven by this study. Since CD8α+ DCs preferentially prime CTLs, this would explain the increased CTL response we observed. Although the existence of these cells as a result of DNA immunization with beta-defensin–antigen fusion constructs has not been reported yet, the presence of such cells would clarify many of the hitherto-unexplained findings by ourselves and others.

In vaccinated and BoHV-1-challenged cattle, the addition of BNBD3 as a fusion construct modified the immune response; VN antibody levels were maintained, and the numbers of tgD-specific IFN-γ-secreting cells, particularly CD8+ IFN-γ+ cells and CD8+ IFN-γ− CD25+ CTLs, were increased. Thus, BNBD3 promoted a predominantly Th1 response that included induction of CD8+ CTLs. While it is generally accepted that Th1 immune responses drive cellular immunity and Th2 immune responses preferentially drive humoral immunity (79, 80), and indeed the Th1-polarized/biased cellular response that we observed fits neatly into this model, in this context, it was puzzling that the VN antibody was maintained. Our data may be explained, however, by the findings of others. Reports have suggested that while Th1-type cytotoxic

TABLE 2 Flow cytometric analysis of bovine CD8+ IFN-γ+ and CD8+ IFN-γ− CD25+ CTLs

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Treatment</th>
<th>% CTLs</th>
<th>CD8+ IFN-γ+</th>
<th>CD8+ IFN-γ− CD25+</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>pMASIA-tgD</td>
<td>19.7</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>pMASIA-tgD</td>
<td>16.3</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>pMASIA-BNBD3-tgD</td>
<td>36.5</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>pMASIA-BNBD3-tgD</td>
<td>36.4</td>
<td>22.7</td>
<td></td>
</tr>
</tbody>
</table>

FIG 7 Effect of vaccination on the phenotypes of IFN-γ-secreting T cell subpopulations in PBMCs of calves vaccinated with plasmids encoding tgD and/or BNBD3, and challenged with BoHV-1. PBMCs were isolated from the peripheral blood of two animals from each of the pMASIA-tgD, pMASIA-BNBD3-tgD, and negative-control groups prior to challenge and on day 16 after challenge (2 animals per group). CD4-depleted (CD4−), CD8-depleted (CD8−), CD4+, and CD8+ subsets were isolated from PBMCs by MACS, and the homogeneity of the resulting CD4− and CD8− cell populations was determined by FACS. (a to f) Dot plots are for one animal (96) from the pMASIA-BNBD3-tgD group on day 16 postchallenge and are representative of all depletions. (a) PBMCs recognized by CD4 MAb. (b) CD4+ population recognized by CD4 MAb. (c) CD4− population recognized by CD4 MAb. (d) PBMCs recognized by CD8 MAb. (e) CD8+ population recognized by CD8 MAb. (f) CD8− population recognized by CD8 MAb. (g and i) Frequencies of IFN-γ-secreting cells in the PBMCs and CD8+, CD8−, and CD8− CD25− cells were determined by FACS (g) and from the pMASIA-BNBD3-tgD vaccinated group (i). (h and j) Frequencies of IFN-γ-secreting cells in the PBMCs and CD8+, CD8−, and CD8− CD25− cells were determined by FACS (h) and from the pMASIA-BNBD3-tgD vaccinated group (j). The number of IFN-γ-secreting cells per 10⁶ cells was calculated as the difference between the number of spots in the tgD-stimulated wells and the number of spots in the medium control wells. FSC, forward scatter.
Kines exert an overall negative effect on systemic humoral responses (79, 81), they can have a positive effect on the magnitude of neutralizing antibody responses (82), and that neutralizing antibody responses can occur concurrent with induction of Th1-polarized responses (83–85). In particular, our results (VN antibodies concurrent with induction of CTL) with this defensin-antigen fusion construct in cattle bears striking similarity to the results obtained when in the mouse model, an experimental DNA vaccine expressing murine beta-defensin 2 (mBD2) as a fusion with the gp120 antigen of HIV-1 induced systemic and mucosal CTLs and neutralizing antibody to the HIV-1 envelope protein in i.d. immunized mice (28). Although the exact mechanism was not determined, the authors theorized that the immunomodulatory effect of the vaccine could have been due to the previously discovered chemotactic nature of mBD2 for iDCs (36). Additionally, the authors suggested that beta-defensin might have targeted receptors on antigen-presenting cells, induced expression of costimulatory molecules, and/or induced production of proinflammatory cytokines, particularly by iDCs (28).

More recently, a beta-defensin adjuvinating strategy was evaluated in chickens, whereby birds were immunized intramuscularly (i.m.) with a DNA vaccine encoding a fusion construct of the mature form of avian beta-defensin 1 (AvBD1) with the VP2 protein of infectious bursal disease virus (IBDV) (29). Unlike our results in cattle, in this study the plasmid encoding the fusion construct induced significantly greater antibody responses than the plasmid encoding the antigen (VP2) alone. The greater antibody response might have been due to the route of administration, as higher humoral responses have been observed when DNA vaccines have been delivered i.m. (86), or to differences in species or the two beta-defensins. Comparable to the augmented cellular responses, including increased numbers of CD8+ cells, that we observed in cattle, in this avian model increased percentages of CD3, CD4, and CD8 T cells were observed in birds immunized with the fusion construct. After challenge with IBDV, the 10 chickens immunized with the AvBD1 fusion construct were protected, while in the group given the DNA vaccine encoding VP2 alone, eight out of 10 were protected. Despite the modest improvements in humoral and cellular immunity and protection from IBDV, the authors concluded that AvBD1 in a fusion construct enhanced VP2 DNA vaccine immunity and protection from IBDV. The authors further suggested that the effect of AvBD1 on improved CMI responses may have been responsible for the protection induced by the fusion construct, particularly since CMI and specifically T cell responses had been shown to be important in protection from IBDV infection.

In cattle, protective vaccination against BoHV-1 has been described for commercially available MLV or KV BoHV-1 vaccines. As such, it has been defined as an observed reduction in clinical signs such as decreased virus shedding, lowered temperature, and decreased nasal secretions (87). Following challenge with BoHV-1, we observed a reduction in the clinical signs of infection in calves vaccinated with the fusion construct pMASIA-BNBD3-tgD. Contrary to what was observed in the avian model, the addition of beta-defensin gave protection equivalent to, but not better than, what was observed in the group given the DNA vaccine encoding VP2 alone, eight out of 10 were protected. Despite the modest improvements in humoral and cellular immunity and protection from BoHV-1, the authors concluded that AvBD1 in a fusion construct enhanced VP2 DNA vaccine immunity and protection from BoHV-1. This was surprising in light of the improvements seen in the avian study and because protective vaccination against BoHV-1 has also been associated with increased CMI responses, particularly those in the form of increased IFN-γ production (87). Given that humoral immunity was not enhanced in calves vaccinated with pMASIA-BNBD3-tgD compared to those vaccinated with pMASIA-tgD and that inefficient humoral immune responses have been implicated in a lack of protection from BoHV-1 challenge (reviewed in reference 88), this does suggest that the humoral immune responses were not high enough and that the improved cellular immunity induced by BNBD3 was not sufficient to result in enhanced protection from BoHV-1.

FIG 8 Clinical signs and virus shedding after BoHV-1 challenge. Eight- to 9-month-old BoHV-1-seronegative Angus and Hereford crossbred calves were immunized three times i.d. by needle-free injection with 0.75 mg plasmid, followed by BoHV-1 challenge 1 month after the last immunization (6 animals per group). (a) Mean (geometric) virus shedding in nasal secretions of calves challenged with BoHV-1. (b) Mean weight change. (c) Mean rectal temperatures. *, P < 0.05; **, P < 0.01.
Here we tested our hypothesis that inclusion of an iDC-chemotactic beta-defensin either encoded on a separate plasmid or as a fusion construct with antigen in a DNA vaccine would improve the efficacy of a BoHV-1 DNA vaccine for cattle. In summary, delivery of BNBD3 by separate plasmid did not enhance immune responses in cattle, while the addition of BNBD3 as a fusion construct modulated the immune response to the DNA vaccine, resulting in increased cell-mediated immunity. Protection against BoHV-1 was afforded to an equal extent by DNA vaccines encoding tGd alone or as a fusion with BNBD3. Taken together, from our results and those of others regarding the effect of beta-defensins on DNA vaccines, some patterns emerge that are worth noting, as they suggest directions where further study could be productive. With respect to humoral responses, systemic antigen-specific IgG responses appear to vary with the antigen, the route of delivery, and the species, while CMI responses appear to be improved by beta-defensin regardless of the nature of these factors.

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