We developed *Lactobacillus casei* bacterial ghosts (BGs) as vehicles for delivering DNA vaccines and analyzed their effects on immune responses. Uptake of the plasmids encoding the enhanced green fluorescent protein (pCI-EGFP) and BGs loaded with pCI-EGFP by macrophages was investigated using fluorescence microscopy and flow cytometry. The results showed that pCI-EGFP-loaded *L. casei* BGs were efficiently taken up by macrophages.

*Lactobacillus casei* BGs loaded with plasmids encoding VP6 protein of PoRV (pCI-PoRV-VP6) significantly upregulated the mRNA expression of interleukin (IL)-1β, IL-10, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1), Mannose receptor (CD206) toll-like receptor (TLR)-2, TLR4, and TLR9 in macrophages. The levels of markers of M1 polarization (IL-10 and TNF-α) and M2 polarization (Arg-1 and CD206) were increased in macrophages incubated with pCI-PoRV-VP6-loaded BGs compared with the control group. The results of the enzyme-linked immunosorbent assay showed that the secretion of IL-1β, IL-10, and TNF-α in macrophages was significantly upregulated compared with the control group. Flow cytometry demonstrated that *L. casei* BGs loaded with pCI-PoRV-VP6 promoted the maturation of dendritic cells (DCs). Following incubation with pCI-PoRV-VP6-loaded BGs, the mRNA expression levels of IL-1β, IL-6 and interferon (IFN)-γ in DCs were significantly increased. ELISA assay showed the secretion of the IL-1β, IL-6, IFN-γ IL-10 and TNF-α in DCs were upregulated significantly. Thus, *L. casei* BGs promoted the maturation and activation of DCs. We analyzed the stimulatory capacity of DCs in a mixed lymphocyte reaction with allogeneic T cells. T cell proliferation increased upon incubation with DCs stimulated by BGs. After immunizing mice with BGs loaded with pCI-PoRV-VP6, the specific IgG levels in the serum were higher than those elicited by BGs loaded with pCI-PoRV-VP6. BGs loaded with pCI-PoRV-VP6 on Th1 and Th2 cytokines polarized T cells.
INTRODUCTION

DNA vaccines are a powerful technology that has revolutionized vaccine research (1). These vaccines have many advantages over conventional vaccines. However, DNA vaccines show poor immunogenicity compared with traditional vaccines. Furthermore, they cannot effectively target antigen-presenting cells (APCs) and require high plasmid dosages (2, 3). Therefore, improving the immune response of DNA vaccines is a key factor determining the immune response.

Bacterial ghosts (BGs) bacterial shells without contents that preserve the entire surface structure of bacteria, including the outer membrane proteins, and show potential as vaccine candidates (4). In addition, BGs can be loaded with DNA, proteins, and drugs and exploited as delivery systems (5–7). Because BGs contain many Toll-like receptor (TLR) agonists, they can effectively target APCs and elicit potent immune responses against BG-delivered antigens (8–10). However, the use of BGs prepared from pathogenic bacteria poses safety concerns because the bacteria membranes cannot be completely split during the preparation of BGs, resulting in their inherent tendency to revert to pathogenicity. Moreover, lipopolysaccharide (LPS) and endotoxic effects in BG envelopes cannot be eliminated (11). Currently, all reported BGs have been prepared from pathogenic bacteria, and most are made of gram-negative bacteria (12, 13). Therefore, safe host bacteria must be used to develop BGs for delivery system applications.

Some studies showed that BGs can present antigens to regulate the polarization of T helper 1 (Th1) and T helper 2 (Th2) immune responses (14). Other studies demonstrated that BGs effectively stimulate macrophages and monocytes and polarize the immune response towards Th1 (15). Dendritic cells (DCs) are APCs that modulate T-cell responses. These cells can produce effective immune responses and establish immunological memory (16, 17). Mature DCs not only induce the activation and proliferation of T cells, but also play a role in the initiation of immunological tolerance (18, 19). Macrophages are APCs responsible for processing antigens and presenting them to antigen-specific T cells. Activated macrophages induce the expression of costimulatory molecules and cytokines that promote continued stimulation of T cells and the induction of adaptive immunity, linking innate and adaptive immunity (20).

Successful delivery of DNA to APCs requires a suitable delivery system and adequate antigen formulation. BGs have been widely used as an adjuvant and a delivery carrier for many bacterial and viral antigens (21, 22). In this study, we evaluated the ability of Lactobacillus casei BGs to deliver DNA vaccines in vivo and in vitro. We examined the efficacy of L. casei BGs in mediating transgene expression in macrophages, interaction of L. casei BGs with macrophages, and induction of cytokine expression. The interaction of DCs with L. casei BGs and their influence on DC maturation and cytokine production were further analyzed. These findings improve the understanding of the function of BGs in presenting DNA to APCs and initiation and regulation of immune responses. Materials and Methods.

Ethics Statement

This trial was conducted in accordance with the requirements of the regulations governing laboratory animals and the Charter of the Ethics Committee for Laboratory Animals of the Northeast Agricultural University.

Bacterial Strains and Plasmids

The RAW264.7 cell line was purchased from the China Center for Type Culture Collection (Wuhan, China). Lactobacillus casei ATCC 393 was kindly provided by the Netherlands NIZO Institute (Ede, Netherlands). The recombinant strain pPG-2-hocb/L. casei 393 (23) and plasmid pCI-PoRV-VP6 were constructed and stored in our laboratory. Total RNA was extracted from porcine Rubulavirus (PoRV) (JL94 strain). After reverse transcription, VP6 (encoding VP6 of PoRV) was amplified from the product by polymerase chain reaction (PCR) using the forward primer 5’-GCTTAGCATA CCATGGAGGTTCTGTACTCA-3’ and reverse primer 5’- GTCGACTCATTTAATCAACATGCT-3’. The gene fragment was 1275 bp in length. The VP6 gene was cut with the restriction enzymes Nhe I and Sal I and inserted into the pCI-neo vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Recombinant plasmids were transformed into Escherichia coli DH5α cells. The plasmids were extracted using kits for rapid extraction of plasmid DNA (Sigma, St. Louis, MO, USA) and stored at -20°C.

Animals

BALB/c mice (specific pathogen-free) were purchased from Changsheng Biotechnology Limited (Liaoning, China). All animal experiments were performed and animals were maintained according to the Ethical Committee for Animal Sciences of Heilongjiang Province and international recommendations for animal welfare.

Preparation of Bacterial Ghosts

The recombinants were inoculated in 50 mL MRS medium containing 10 µg/mL of chloramphenicol (Cm) and cultivated at 37°C for 12 h. 2% levulose was then added to the culture and cultivated at 37°C for 36 h. L. casei BGs were produced by expressing the phage-derived holin. PH of the culture was regulated and maintained ranging from 5.5 to 6.5 with 2 mol/L NaOH every 12 h. When OD600 of the culture did not decline,
the lysed cells were collected by centrifugation at 5000× g for 20 min and treated with gentamycin (50 mg/mL) and streptomycin (100 mg/mL) to kill the surviving bacteria. Subsequently the BGs were washed twice with phosphate-buffered saline (PBS, pH 7.4) and resuspended in 5% sucrose solution. The BGs were lyophilized and stored at 4°C.

**Loading BGs With DNA Plasmid**

Plasmid DNA was loaded into the BGs via diffusion through lysis holes. Lyophilized BGs (30 mg) were suspended in 10 mM sodium acetate, 100 mM NaCl, and 10 mM HEPES (pH 7.5) containing pET30a/PoRV/VP6 and pEGFP-C1 (final concentration 1 mg/mL). The samples were mixed with CaCl₂ (final concentration, 25 mM) and agitated at 37°C for 15 min. Subsequently, the BGs were centrifuged at 10,000 × g for 20 min; the pellets were washed once and resuspended in PBS.

**Transfection Experiments**

BALB/c mice were administered Dulbecco’s Modified Eagle Medium (DMEM) by intraperitoneal injection and then the ascites fluid was extracted. Peritoneal macrophages in the ascites fluid were centrifuged at 2000 × g for 20 min. Both peritoneal macrophages and RAW264.7 macrophages were cultured in DMEM containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA). For transfection experiments, RAW264.7 macrophages and peritoneal macrophages were inoculated into 24-well plates (1 × 10⁵/well) and incubated with pCI-EGFP (1 mg/mL) and BGs loaded with pCI-EGFP (1000 bacteria/cell) for 2 h for attachment to and processing by the macrophages. The cells were washed with PBS to remove unabsorbed BGs and then incubated at 37°C for another 48 h. EGFP expression and transfection efficiency were determined by performing fluorescence microscopy and flow cytometry, respectively, with a FACS caliber (BD Biosciences, Franklin Lakes, NJ, USA). Untreated cells were used as controls.

**Real-Time PCR Assay of Marker Expression by Macrophages**

RAW264.7 macrophages and peritoneal macrophages were inoculated into 24-well plates (1 × 10⁵/well) and cultured at 37°C for 12 h. The cells were incubated with plasmid pCI-PoRV-VP6 (1 mg/mL), BGs (1000 bacteria/cell), and BGs loaded with pCI-PoRV-VP6 (1000 bacteria/cell) at 37°C for 2 h. After washing three times with PBS, the cells were cultured in fresh medium at 37°C. After cultivation for 6, 12, 18, 24, and 30 h, the IL-1β, IL-10, and TNF-α levels in the supernatant were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Meimian, Jiangsu, China) according to the manufacturer’s instructions.

**ELISA of Cytokine Secretion in Macrophages**

RAW264.7 macrophages and peritoneal macrophages were inoculated into 24-well plates (1 × 10⁵/well) and cultured at 37°C for 12 h. Subsequently, the cells were incubated with plasmid pCI-PoRV-VP6 (1 mg/mL), BGs (1000 bacteria/cell), and pCI-PoRV-VP6-loaded BGs (1000 bacteria/cell) at 37°C for 2 h. After washing three times with PBS, the cells were cultured in fresh medium at 37°C. After cultivation for 6, 12, 18, 24, and 30 h, the IL-1β, IL-10, and TNF-α levels in the supernatant were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Meimian, Jiangsu, China) according to the manufacturer’s instructions.

**Preparation of DCs Derived From Bone Marrow Cells**

DCs derived from bone marrow cells were obtained from the femurs of mice and harvested by density gradient centrifugation (24, 25). Interphase cells were isolated and washed three times with PBS. The DCs were cultured in 6-well plates (5 × 10⁶ cells/well) in RPMI-1640 medium (Sigma) containing 10% fetal calf serum (PeproTech, Rocky Hill, NJ, USA), streptomycin (100 µg/mL) (PeproTech), 40 ng/mL granulocyte-macrophage colony-stimulating factor (PeproTech), and 20 ng/mL IL-4 (PeproTech). Unattached cells were removed by washing after 3 h, and the attached cells were cultured. The culture medium was replaced with fresh medium containing the same concentration of cytokines every two days. After 7 days, nonadherent cells were collected, inoculated into six-well plates, and cultured in incomplete RPMI-1640 medium supplemented with IL-4 (10 ng/mL) and granulocyte-macrophage colony-stimulating factor (20 ng/mL) for another 2 days. The DCs were harvested for further experiments.

**Flow Cytometry Assay of Marker Expression by Mature DCs**

For stimulation experiments, DCs (1 × 10⁶/mL) were inoculated into six-well plates with RPMI-1640 medium and incubated with pCI-PoRV-VP6 (1 mg/mL), BGs loaded with pCI-PoRV-VP6 (1000 bacteria/cell), or LPS (1 µg/mL) for 24 h. The treated DCs were collected and washed twice with staining buffer (0.1% NaN₃ and 0.5% fetal calf serum in PBS), followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse major histocompatibility complex (MHC) class II, CD80, and CD86 antibodies (Sigma, St. Louis, MO, USA) at 4°C for 30 min. Fluorescein isothiocyanate-conjugated isotype control antibodies were used as negative controls. The cells were analyzed using a FACS Caliber. The results were analyzed using BD FACSDIVA software (BD Biosciences).
Real-Time PCR Assay of Marker Expression by DCs
DC cultures stimulated with pCI-PoRV-VP6 (1 mg/mL), BGs loaded with pCI-PoRV-VP6 (1000 bacteria/cell), or LPS (1 μg/mL) for 24 h were harvested. Total RNA was extracted as described in 2.7. The mRNA levels of IL-1β, IL-6, IFN-γ IL-10 and TNF-α were quantified using quantitative reverse transcription PCR (RT-qPCR). The primers used for the target genes are listed in Table 1. To analyze gene expression, cDNA was amplified using Power SYBR Green PCR Master Mix (24). Each gene was amplified in triplicate, and the levels of cytokine mRNA (normalized to that of GAPDH) were analyzed using the 2-ΔΔCT method as described previously (24).

ELISA of Cytokine Secretion by DCs
Cytokine secretion by DCs was analyzed using ELISA. DC cultures stimulated with pCI-PoRV-VP6 (1 mg/mL), pCI-PoRV-VP6-loaded BGs (1000 bacteria/cell), or LPS (1 μg/mL) for 24 h were harvested. The levels of IL-1β, IL-6, IFN-γ IL-10 and TNF-α in the cell supernatant were detected using an ELISA kit (Meimian, Jiangsu, China) according to the manufacturer’s instructions.

Mixed Leukocyte Reaction
DCs were cultured for 7 days and then incubated with pCI-PoRV-VP6 (1 mg/mL), pCI-PoRV-VP6 loaded with BGs (1000 particles/cell), or LPS (100 ng/well) for 24 h. Mitomycin C (50 mg/mL) (Merck KgaA, Darmstadt, Germany) was added to the cultured DCs and then incubated at 2 h for 37°C. The treated DCs were washed three times with PBS. T lymphocytes (1 × 10⁶/mL) were cultured in triplicate in 96-well culture plates and then mixed with cultured DCs at ratios of 1:1, 1:10, and 1:100. Splenocytes stimulated by ConA (5 μg/mL) were used as positive controls, splenocytes were used as negative controls, and 1640 culture medium was used as a blank control.Mixed cells were cultured for 72 h at 37°C in 96-well plates. Untreated T-cells served as negative controls. T cells proliferation was measured in an MTT assay (26). The stimulation index (SI) was calculated as the mean absorbance by subtracting the OD₄₀₀ from the OD₅₇₀ after subtracting the absorbance reading for untreated cells at each wavelength.

Immunization
Groups of eight-week-old BALB/c mice (n = 5) were immunized on days 0, 14, and 28 via the oral, intramuscular injection, or intraperitoneal injection route with pCI-PoRV-VP6 alone (80 μg), BGs loaded with pCI-PoRV-VP6 (10⁹ colony-forming units, containing 80 μg of DNA), unloaded BGs, and PBS, respectively. Serum samples were collected from the mice on days 1, 7, 14, 21, 28, 35, and 42 after immunization and stored at -20°C until ELISA.

ELISA of IgG in Serum
Specific antibodies in the serum were analyzed by using ELISA. VP6 protein was coated at 2 μg per well in polystyrene microtiter plates at 4°C for 12 h (purified VP6 protein expressed in E. coli was prepared in our laboratory). The ELISA plates were washed with PBS containing 1% Tween-20 three times and then blocked with PBS containing 5% skim milk at 37°C for 3 h. The serum was serially diluted and incubated at 37°C for 1 h after washing three times. The samples were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) diluted 1:3000, washed, and mixed with 100 μL of o-phenylene diamine dihydrochloride substrate (Sigma). The OD₄⁹₀ was measured.

Lymphoproliferation Assay
Mice from each group were sacrificed at 14 days after the last immunization, and their spleens were removed aseptically. The spleens were minced and then pressed through a fine copper wire screen mesh (100 μm). The sample was collected by centrifugation 456 g for 10 min, followed by incubation in 15 mL of 0.7% NH₄Cl at 37°C for 10 min. The liquid was then centrifuged at 465 g for 10 min. The deposit was washed three times with RPMI-1640 medium after discarding the supernatant. The splenocytes were diluted to 1 × 10⁶/mL in RPMI-1640 medium containing 10% fetal bovine serum and then co-cultured with VP6 protein (0.5 or 5 μg/mL) at 37°C for 72 h. Splenocytes stimulated with ConA (5 μg/mL) and untreated cells served as positive and negative controls, respectively. Cell proliferation was measured in an MTT assay (26). The SI was calculated as the mean absorbance by subtracting the OD₄₆₀₀ from the OD₅₇₀ after subtracting the absorbance reading for untreated cells at each wavelength.

Flow Cytometry Analysis of CD4⁺ and CD8⁺ T Cells
Spleen lymphocytes from each group were prepared as described in 2.16. Splenocytes were diluted to 1 × 10⁶/mL and incubated with antibodies against anti-CD3-fluorescein isothiocyanate, anti-CD4-biotin (BIOT, Hangzhou, China), and anti-CD8-phycocerythrin (Sigma) in the dark at 4°C for 30 min. After washing three times with PBS, all samples were analyzed by flow cytometry using a FACS Caliber. The results were analyzed using BD FACSDIVA software.

Th1 and Th2 Cytokine Analysis
Serum samples were collected at 24 h after immunization. Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) levels were analyzed using an ELISA kit (Meimian, China) according to the manufacturer’s instructions.

Statistical Analysis
The data are expressed as the mean ± standard deviation (SD). Statistical data were analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA) for analysis of variance.*P < 0.05, **P < 0.01, ***P < 0.001; #P < 0.05, ##P < 0.01, ###P < 0.001. were considered to indicate statistically significant differences.

RESULTS
EGFP Expression in Macrophages by Ghost-Mediated Transfection
Both peritoneal macrophages and RAW264.7 cells were incubated with pCI-EGFP and pCI-EGFP-loaded BGs,
respective. Uptake of pCI-EGFP and BGs loaded with pCI-EGFP by macrophages was investigated using fluorescence microscopy (Figure 1) and flow cytometry (Figure 2). Most RAW264.7 cells (Figure 1C) and peritoneal macrophages (Figure 1F) exhibited green fluorescence in their cytoplasm after incubation with pCI-EGFP-loaded BGs. Only a small amount of green fluorescence was observed in RAW264.7 cells (Figure 1E) and peritoneal macrophages (Figure 1B) incubated with pCI-EGFP. No green fluorescence was observed in untreated cells (Figures 1A, D). We further investigated EGFP expression in the macrophages using flow cytometry. The fluorescence observed by flow cytometry in RAW264.7 and peritoneal macrophages was increased from 1.4% to 7.2% (Figures 2B, C) and 1.8% to 57.6% (Figures 2E, F), respectively, confirming the results of fluorescence microscopy. These results suggest that pCI-EGFP-loaded L. casei BGs can be taken up more efficiently by macrophages. The more efficient expression of EGFP was mediated by L. casei BGs compared to in control cells.

**BGs Regulate mRNA Expression of Cytokines and TLRs in Macrophages**

The potential of BGs to regulate the mRNA expression of TLRs and cytokines in macrophages was investigated using RT-qPCR. The RAW264.7 cells incubated with pCI-PoRV-VP6-loaded BGs expressed higher levels of IL-1β, IL-10, TNF-α, iNOS, TLR2, and TLR9 compared to cells incubated with pCI-PoRV-VP6 (Figures 3, 4). The expression of IL-1β, IL-10, TNF-α, iNOS, TLR2, TLR4, and TLR9 peaked at 24, 30, 18, 30, and 18 h, respectively (Figures 3A, C, F, G and Figures 4A, C, E). After incubation with pCI-PoRV-VP6-loaded BGs, the mRNA expression levels of IL-1β, IL-10, TNF-α, iNOS, TLR2, and TLR9 in peritoneal macrophages were significantly increased. The expression of IL-1β, IL-10, TNF-α, iNOS, TLR2, TLR4, and TLR9 in peritoneal macrophages peaked at 12, 6, 12, 24, 6, 18, and 18 h, respectively (Figures 3B, D, F, H and Figures 4B, D, H). These results demonstrate that L. casei BGs significantly upregulated the mRNA expression levels of most inflammatory cytokines and TLRs in macrophages. The expression levels of different inflammatory cytokines and TLRs peaked at different times.

The markers of M1 polarization (Figures 3C–F) and M2 polarization (Figures 3G, I, K, L) were all higher in macrophages incubated with pCI-PoRV-VP6-loaded BGs compared to in control cells. These results indicate that both M1-type and M2-type polarization was induced by BGs, with greater effects on M1-type polarization.

**Analysis of Cytokines in Macrophages by ELISA**

Cytokine secretion by macrophages was analyzed using ELISA. After incubation with pCI-PoRV-VP6-loaded BGs, the secretion of IL-1β, IL-10, and TNF-α in RAW264.7 cells peaked at 24, 30, and 24 h, respectively (Figures 5A–C). The secretion of IL-1β, IL-10, and TNF-α in peritoneal macrophages peaked at 24, 6,
and 12 h, respectively (Figures 5D–F). These results show that *L. casei* BGs significantly upregulated the levels of most of the evaluated inflammatory cytokines in macrophages.

**Lactobacillus casei** BGs Promote DC Maturation

The antigen-presenting co-stimulatory molecules CD80, CD86, and MHC class II are surface markers of mature DCs. To analyze the influence of *L. casei* BGs on the maturation of bone marrow derived DCs, CD80, CD86, and MHC class II expression in DCs was analyzed using flow cytometry. In untreated DCs, the positivity rates of CD86, CD80, and MHC-II were 3.2% (Figure 6A), 0% (Figure 6E), and 1% (Figure 6I), respectively. After pCI-PoRV-VP6 stimulation of DCs, the positive rates of CD86, CD80, and MHC-II were 3.7% (Figure 6B), 1.5% (Figure 6F), and 37.4% (Figure 6J), respectively. After the DCs were stimulated by DNA-packed *L. casei* BGs, the positive rates of CD86, CD80, and MHC-II were 22.4% (Figure 6C), 12.0% (Figure 6G), and 60.5% (Figure 6K), respectively. In DCs treated with LPS, the positivity rates of CD86, CD80, and MHC-II were 19.8% (Figure 6D), 7.0% (Figure 6H), and 42.1% (Figure 6L), respectively. Preincubation with DNA-loaded BGs for 24 h led to increased expression of MHC class II in DCs treated with DNA (Figures 6I, K). The expression of CD80 and CD86 was also upregulated after treatment with DNA-loaded *L. casei* BGs compared to that in DCs treated with DNA (Figures 6B, C, F, G). Stimulation with *L. casei* BGs significantly enhanced MHC-II, CD80, and CD86 expression, indicating that *L. casei* BGs promoted DC maturation.

**Lactobacillus casei** BGs Induce Cytokine mRNA Expression in DCs

To analyze the effect of *L. casei* BGs on the activation of bone marrow-derived DCs, DCs were pre-incubated with DNA and DNA-loaded BGs for 24 h. The mRNA expression levels of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ were investigated using RT-qPCR (Figure 7). Lower levels of inflammatory cytokines were observed in untreated cells. Following incubation with DNA-loaded BGs, the mRNA expression levels of IL-1β, IL-6 and IFN-γ inflammatory cytokines in DCs increased significantly compared to those in DCs incubated with DNA. The mRNA expression of IL-10 and TNF-α were also increased, but it was inapparent compared to those in the DCs incubated with DNA. These results showed that *L. casei* BGs upregulated the levels of most of these inflammatory cytokines in DCs significantly.

**Analysis of Cytokines in DCs by ELISA**

The cytokine secretion levels of TNF-α, IL-1β, IL-6, IL-10, and IFN-γ in DCs were analyzed using ELISA. Following incubation with DNA-loaded BGs, the secretion levels of all inflammatory cytokines in DCs were increased significantly compared to those in DCs incubated with DNA (Figure 8). These results demonstrate that incubation of immature DCs with DNA-loaded *L. casei* BGs resulted in the maturation and activation of DCs. Thus, *L. casei* BGs...
FIGURE 3 | mRNA expression of inflammatory factors in RAW264.7 cells and peritoneal macrophages. RAW264.7 cells and peritoneal macrophages were incubated with plasmid pCI–PoRV–VP6 (1 mg/mL) and bacterial ghosts (BGs) loaded with pCI–PoRV–VP6 (1000 bacteria/cell), respectively. Total RNA was extracted at different times, and the mRNA expression of inflammatory factors was quantified using RT–qPCR. mRNA expression of (A) IL–1β in RAW264.7 cells, (B) IL–1β in peritoneal macrophages, (C) IL–10 in RAW264.7 cells, (D) IL–10 in peritoneal macrophages, (E) TNF–α in RAW264.7 cells, (F) TNF–α in peritoneal macrophages, (G) iNOS in RAW264.7 cells, (H) iNOS in peritoneal macrophages, (I) Arg–1 in RAW264.7 cells, (J) Arg–1 in peritoneal macrophages, (K) CD206 in RAW264.7 cells, and (L) CD206 in peritoneal macrophages. Values are expressed as the mean ± SD of four individual experiments. Significance of differences between the LPS, BG+DNA, DNA, and Macrophage culture medium (Med) groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates a significant differences between BG+DNA, DNA, and Med groups: #P < 0.05, ##P < 0.01, ###P < 0.001. n.s., no significance.
BGs significantly upregulated the levels of most of inflammatory cytokines in DCs. These results demonstrate that incubation of immature DCs with DNA-loaded *L. casei* BGs resulted in the maturation and activation of DCs. *Lactobacillus casei* BGs activated DCs to produce both anti-inflammatory and proinflammatory cytokines, which may improve immune responses using *L. casei* BGs as a delivery system for DNA vaccines.

**Lactobacillus casei** BGs Increase Allostimulatory Capacity of DCs

A remarkable property of DCs is their ability to stimulate naïve T cells and present antigens in vivo. This ability can be analyzed in vitro in a mixed leukocyte reaction. DCs were cultured for 7 days and then incubated with LPS, ConA, plasmid DNA, or plasmid DNA-loaded BGs for 24 h. We then analyzed the stimulatory capacity of DCs in the mixed leukocyte reaction with allogeneic T cells. DCs treated with DNA-loaded BGs or LPS significantly activated allogenic T cells at APC: T ratios of 1:1 and 10:1 (Figure 9) compared to in DCs treated with DNA. The results showed that T cell proliferation responses increased when DCs were stimulated with BGs. Thus, the effect of DCs on maturing T cells was enhanced following incubation with DNA-loaded BGs. Mature DCs stimulate the activation and proliferation of naïve T cells in vivo. Our findings demonstrate that *L. casei* BGs efficiently...
promoted the maturation of DCs and improved the allostimulatory capacity of DCs.

**Determination of Specific IgG Antibody**

Groups of mice were immunized orally ([Figure 10A](#)), intramuscularly ([Figure 10B](#)), or intraperitoneally ([Figure 10C](#)) with pCI-PoRV-VP6, BGs loaded with pCI-PoRV-VP6, unloaded BGs, and PBS, respectively. Serum samples were collected on days 1, 7, 14, 21, 28, 35, 42, and 49. Significant levels of specific anti-VP6 IgG were observed after immunization with pCI-PoRV-VP6 or BGs loaded with pCI-PoRV-VP6 compared to the group immunized with PBS or BGs ([Figures 10 A–C](#)). The specific IgG levels in serum samples elicited by DNA-loaded BGs were significantly higher at most of time points than those elicited by BGs administered with pCI-PoRV-VP6 ([Figure 10](#)). Group of DNA-loaded BGs showed higher IgG levels than group of DNA at most of time points except for a minority of the time points, for example day 14.

**Lymphoproliferation Assay**

Lymphocyte lymphoproliferation was examined to investigate the antigen-specific cell-mediated immune response. The lymphocytes of immunized mice proliferated ([Figure 11](#)). The stimulation index difference was significant in lymphocytes of the immunized mice incubated with VP6 protein at 5 µg/mL ([Figure 11](#)). No significant difference was observed in the group immunized via the oral or intramuscular route stimulated with VP6 protein at 5 µg/mL.
Measurement of CD4+ and CD8+ T Cells in Immunized Mice

We analyzed the percentages of CD4+ and CD8+ T cells after isolating splenic lymphocytes from the mice in each group. As shown in Figure 10, vaccination with pCI-PoRV-VP6 or pCI-PoRV-VP6-loaded BGs induced changes in the populations of CD4+ and CD8+ T cells (Figures 12A, B). The proportions of both CD4+ and CD8+ T cells from mice administered with DNA or DNA-loaded BGs increased significantly compared to those in mice administered with PBS or BGs (Figure 12C). These results indicate that pCI-PoRV-VP6-loaded BGs promoted the proliferation of T cells and polarized Th cells into CD4+ T cells to enhance the immune response.

Effect of DNA Loaded by Immune BGs on Th1 and Th2 Cytokines

Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) levels in the serum samples after immunization were evaluated using ELISA. Significant increased levels of IFN-γ were observed after immunization with pCI-PoRV-VP6-loaded BGs compared to those immunized with PBS in the oral group. Significant levels of IFN-γ were observed after immunization with pCI-PoRV-VP6-loaded BGs compared to those in the immunization with pCI-PoRV-VP6, PBS, or BGs in the intramuscular or intraperitoneal injection groups (Figure 13A). Significant levels of IL-4 were observed after immunization with pCI-PoRV-VP6-loaded BGs compared to those following immunization with pCI-PoRV-VP6, PBS, or BGs in the intramuscular injection group (Figure 13B). This result indicates that T cells tended to polarize toward the Th1 type.

DISCUSSION

The use of naked DNA for immunization is a breakthrough in vaccine research. However, DNA possesses poor immunogenicity, and antigens encoded by DNA are not specifically targeted to APCs,
FIGURE 7 | Expression of IL-1β, IL-6, IL-10, TNF-α, and IFN-γ mRNA levels in dendritic cells (DCs). DCs were incubated with pCI-PoRV-VP6 plasmid DNA (1 mg/mL) and bacterial ghosts (BGs) loaded with pCI-PoRV-VP6 plasmid DNA (1000 bacteria/cell) respectively at 37°C for 24 h. Total RNA was extracted. IL-1β, IL-6, IL-10, TNF-α, and IFN-γ mRNA levels were quantified using RT-qPCR. Values are expressed as the mean ± SD of four individual experiments. Significance of differences between the LPS, BG+DNA, DNA, and Med groups are represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between BG+DNA, DNA, and Med groups: aP < 0.05, bP < 0.01, cP < 0.001. #Asterisk indicates significant difference between DNA and Med groups: #P < 0.05, ##P < 0.01, ###P < 0.001. n.s., no significance.

FIGURE 8 | Secretion of IL-1β, IL-6, IL-10, TNF-α, and IFN-γ levels in dendritic cells (DCs). DCs were incubated with pCI-PoRV-VP6 plasmid DNA (1 mg/mL) and bacterial ghosts (BGs) loaded with pCI-PoRV-VP6 plasmid DNA (1000 bacteria/cell) at 37°C for 24 h. Cytokine contents by DCs were analyzed using ELISA. Values are expressed as the mean ± SD of four individual experiments. Significance of differences between the LPS, BG+DNA, DNA, and Med groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between BG+DNA groups, DNA and Med groups: aP < 0.05, bP < 0.01, cP < 0.001. #Asterisk indicates significant difference between DNA and Med groups: #P < 0.05, ##P < 0.01, ###P < 0.001.

FIGURE 9 | Stimulatory effect of dendritic cells (DCs) by mixed lymphocyte reaction. DCs were stimulated with lipopolysaccharide (LPS, 100 ng/well), ConA (0.5 ng/well), plasmid DNA (1 mg/mL), or DNA-loaded bacterial ghosts (BGs, 1000 particles/cell) for 24 h. T lymphocytes were cultured in triplicate in 96-well culture plates and then mixed with the cultured DCs at ratios of 1:1 (A), 10:1 (B) and 100:1 (C). T cell proliferation was measured using an MTT assay, the results are expressed as the stimulation index (SI). Values are expressed as the mean ± SD of five individual experiments. Significance of differences between the LPS, ConA, BG+DNA, DNA, and Med groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between ConA, BG+DNA, DNA, and Med groups: aP < 0.05, bP < 0.01, cP < 0.001. #Asterisk indicates significant difference between, BG+DNA, DNA, and Med groups: #P < 0.05, ##P < 0.01, ###P < 0.001. n.s., no significance.
limiting its mass implementation. Low efficiency can be achieved by using DNA delivery systems or adjuvants (2). Several studies reported that BGs are effective DNA delivery systems. However, the reported BGs are all made of pathogenic bacteria, typically gram-negative bacteria (11–13), resulting in safety concerns. Moreover, LPS in the cell walls of gram-negative bacteria is associated with the BG envelopes, and it is difficult to eliminate their endotoxic effects (10). We used L. casei BGs as a DNA delivery system. Lactic acid bacteria are generally regarded as safe microorganisms. They are widely used in fermentation and are beneficial to animal health (30). The components in the cell walls of lactic acid bacteria can enhance immune responses (11). Moreover, L. casei BGs can be mass-produced by fermentation, reducing labor and costs.

Macrophages play important roles in immune processes. They not only fight inflammation and infection, but also trigger innate immune responses. When macrophages are activated, pinocytic activity and phagocytic capacity are increased. DNA-loaded BGs can be efficiently targeted to macrophages, and approximately 60% of cells express the plasmid-encoded reporter gene EGFP (27). Our in vitro studies showed that DNA-loaded L. casei BGs were efficiently taken up by RAW164.7 macrophages (from 1.4–7.2%) and peritoneal macrophages (from 1.8–57.6%). These results suggest L. casei BGs can activate macrophages to enhance their phagocytic capacities and further increase the transfection efficiency of DNA delivery. Macrophages play a role in immune responses by releasing inflammatory factors (nitric oxide) and proinflammatory factors (TNF-α and IL-1β) (31). A major characteristic of polarized and activated macrophages is differential cytokine production. Activated M1 phenotypes of polarized type I responses typically produce IL-12 and TNF-α. In contrast, the M2 phenotypes is associated with IL-10 production (32). Different cytokines integrate into M1 and M2 macrophages to attract T regulatory cells or Th1 and Th2 cells to induce polarized T cell responses. In this study, DNA-loaded L. casei BGs significantly upregulated the mRNA expression levels of IL-1β, IL-10, TNF-α, iNOS, Arg-1, and CD206 in RAW264.7 cells and peritoneal macrophages, supporting that L. casei BGs effectively activated macrophages, and DNA-loaded L. casei BGs upregulated IFN-γ and IL-4 cytokine levels, which are secreted by Th1 and Th2 cells. These results suggest L. casei BGs regulate the balance of activated M1 and M2 macrophages and that L. casei BGs simultaneously induce Th1- and Th2-type responses.

TLRs are important membrane receptors that activate macrophages. They are engaged by pathogen-associated molecular patterns in cells to activate signaling pathways, driving innate immune functions that initiate adaptive immune responses (33). We found that L. casei BGs significantly upregulated the mRNA expression of TLR2 and TLR9 in RAW264.7 cells and peritoneal macrophages. However, the mRNA expression levels of TLR4 were decreased. TLR4 expression in macrophages after stimulation by...
polysaccharide from the roots of Actinidia (AEPS) has been reported to be similar to our study (34). Additionally, TLR4 expression in RAW264.7 cells and peritoneal macrophages were downregulated by LPS treatment (35, 36). These results suggest that L. casei BGs regulate TLR expression in macrophages, improving the understanding of the immunomodulatory mechanisms of macrophages via signaling molecules.

DCs are the most effective APCs and initiate primary immune responses by expressing phenotypic markers and functional cytokines (37). Mature DCs are the only cells that can activate naïve T cells (38). We found that L. casei BGs promoted DC maturation and activation. Furthermore, the mRNA expression of IL–6, IL–1β, and IFN–γ was increased in DCs stimulated with DNA–loaded BGs. The mRNA expression of IL–10 and TNF–α were also increased, but it was inapparent compared to those in the DCs incubated with DNA. ELISA assay showed the secretion of the IL–1β, IL–6, IFN–γ IL–10 and TNF–α in DCs were upregulated significantly. IL–1β is a cytokine with many functions and important roles in inducing innate and acquired immune responses (39) {Dinarello, 1996 #20;Dinarello, 1996 #20}. IL–6 is a proinflammatory cytokine (40). TNF–α and IL–10 mainly participate in switching the immunoglobulin class (41). TNF–α also plays an important role in promoting Th2 cells to switch to this class (42). IFN–γ is a key cytokine involved in Th1 polarization and is critical for vaccine–induced protection against pathogens (29). The cytokines released by DCs during activation are important for evaluating stimulated immune responses. Lactobacillus casei BGs induced a wide range of chemokines and cytokines in DCs, demonstrating that efficient immune responses can be induced. Mature T cells can be divided into two main subsets, CD4+ and CD8+, based on their cell–surface identity. The CD4+/CD8− ratio is a direct index of immunity (43). In this study, L. casei BGs considerably increased the CD4+/T cell percentage and CD4+/CD8− ratio 14 days after the last immunization. This result suggests that DNA–loaded L. casei BGs altered the ratio of T–cell subsets and that humoral immunity and cellular immunity were significantly improved, possibly enhancing immune levels.

BGs contain all surface antigens of bacteria required for activating DCs and macrophages in the immune process (44–46). Therefore, humoral and cell–mediated immune responses are stimulated. The results of our in vivo studies showed that

FIGURE 11 | Lymphoproliferation assay. Lymphocytes from the mice immunized by the (A) oral, (B) intramuscular, or (C) intraperitoneal route were prepared 14 days after the last immunization and cultured with VP6 protein of final concentration of 0.5 or 5 μg/mL. The splenocytes stimulated with 5 μg/mL ConA as the positive control. The splenocytes untreated were used as negative controls. Values are expressed as the mean ± SD of five individual experiments. Significance of differences between the PBS, ConA, BG+DNA, DNA, and bacterial ghost (BG) groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between ConA, BG+DNA, DNA, and BG groups: aP < 0.05, bP < 0.01, cP < 0.001. Asterisk indicates significant difference between BG+DNA, DNA, and BG groups: *P < 0.05, **P < 0.01, ***P < 0.001.
FIGURE 12 | CD4⁺ and CD8⁺ T-lymphocytes populations in immunized mice. At 14 days after the last immunization, the splenocytes of mice from each group were harvested. The percentages of (A) CD4⁺ and (B) CD8⁺ T cells were analyzed using flow cytometry. (C) Proportions of CD4⁺/CD8⁺. Values are expressed as the mean ± SD of four individual experiments. Significance of differences between the PBS, bacterial ghost (BG)+DNA, DNA, and BG groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between BG +DNA, DNA, and BG groups: aP < 0.05, bP < 0.01, cP < 0.001. #Asterisk indicates significant difference between DNA and BG groups: #P < 0.05, ##P < 0.01, ###P < 0.001. n.s., no significance.

FIGURE 13 | Effect of DNA loaded by immune BGs on Th1 and Th2 cytokines. Serum samples were collected at 24 h after immunization. (A) IFN-γ level and (B) IL-4 level. Values are expressed as the mean ± SD of four individual experiments. Significance of differences between PBS, BG+DNA, DNA, and BG groups is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Letter indicates significant differences between BG +DNA, DNA, and BG group: aP < 0.05, bP < 0.01, cP < 0.001. #Asterisk indicated significant difference between DNA groups and BG groups: #P < 0.05, ##P < 0.01, ###P < 0.001.
DNA–loaded *L. casei* BGs enhanced humoral and, particularly, cell–mediated immune responses. Thus, *L. casei* BGs containing effective immune stimulants may be useful as immunoadjuvants.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

The protocol (NEAU2018024) was approved by the Ethical Committee for Animal Experiments of Northeast Agricultural University, China.

**AUTHOR CONTRIBUTIONS**

XLY conducted the Real–time PCR, flow cytometry and ELISA assay. LW drafted the manuscript, conducted the Real–time PCR, flow cytometry and ELISA assay. XRY cultured DCs. SSZ prepared BGs. GL and LZ prepared plasmid. JL and XW immunized the experimental animals. HZ, YJ, and WC analyzed the experimental data. YL, LT, and XQ conceived the project. XQ was the grant holder and drafted the manuscript. All authors read, revised, and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

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