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ABSTRACT

Single-stranded viral RNA (ssRNA) was recently identified as the natural ligand for TLR7 and TLR8. ssRNA sequences from viruses, as well as their synthetic analogues stimulate innate immune responses in immune cells from humans and mice, but their immunostimulatory activity has not been investigated in ruminants. In the present investigations, we tested whether synthetic RNA oligoribonucleotides (ORN) can activate immune cells from cattle. In vitro incubation of bovine peripheral blood mononuclear cells (PBMCs) with ORN-induced production of IL-12, IFN-γ and TNF-α. No significant induction of IFN-α was observed. Depletion of CD14+ cells from PBMC abrogated the IL-12 response and consequently the IFN-γ response, suggesting that CD14+ cells are required for PBMC immune activation with ORN. Consistent with these findings, the putative receptors for ORN (TLR7 and TLR8) were expressed at higher levels in the CD14+ fraction than the CD14− PBMC fraction. Pre-treatment of PBMC with bafilomycin (an inhibitor of phagosomal acidification) prior to stimulation with ORN abolished the cytokine responses, confirming that the receptor(s) which mediate the ORN-induced responses are intracellular. These results demonstrate for the first time that the TLR7/8 agonist ORN's have strong immune stimulatory effects in cattle, and suggest that further investigation on the potential of TLR7/8 ligands to activate innate and adaptive immune responses in domestic animals are warranted.

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1. Introduction

The mammalian innate immune system detects invading microbial pathogens via germ-line encoded receptors such as Toll-like receptors (TLRs). Approximately 11 TLRs have been described in mammals and it appears that TLRs distinguish between specific microbial components (Iwasaki and Medzhitov, 2004; Takeda et al., 2003). For example, TLR3, TLR4, TLR7/8 and TLR9 recognize double-stranded viral RNA, bacterial LPS (Poltorak et al., 1998), single-stranded RNA and imidazoquinolines (Hemmi et al., 2002), and CpG DNA (Hemmi et al., 2000), respectively. Activation of TLRs results in secretion of type 1 interferons, proinflammatory cytokines, chemokines and expression of co-stimulatory molecules; events that constitute an innate immune response (Medzhitov and Janeway, 1997). Activation of innate immunity serves to limit the spread of microbial infection, and also plays an important role in the development of antigen-specific immune responses (Iwasaki and Medzhitov, 2004). The realization that TLRs provide a critical link between innate and adaptive immunity (Iwasaki and Medzhitov, 2004) has attracted a lot of interest in the immunobiology of TLR activation.

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TLR7, TLR8 and TLR9 have been grouped in the same subfamily due to their high sequence homology and the structural similarity of their natural ligands (Chuang and Ulevitch, 2000). A lot is known regarding activation of TLR9 with its ligands CpG DNA. Synthetic CpG oligodeoxynucleotides (ODN) bind to TLR9 (Latz et al., 2004a,b; Rutz et al., 2004) and rapidly induce cell signalling pathways including mitogen-activated protein kinases (MAPKs) and NFkB (Hemmi et al., 2000; Yi and Krieg, 1998; Yi et al., 1998) leading to a predominantly Th1 cytokine secretion (Krieg et al., 1998), B proliferation, immunoglobulin secretion, production of IL-6 and IL-10, increased levels of MHC II antigens, and co-stimulatory molecules B7.1 and B7.2 (Klinman et al., 1996; Krieg et al., 1995; Redford et al., 1998) and induction of chemokines IL-8, IP-10 and GM-CSF (Hartmann and Krieg, 1999; Kadowaki et al., 2001). In vivo studies indicate that CpG activates innate immunity and protection against bacteria, viruses and protozoa (Ashkar et al., 2003; Gomis et al., 2003; Gramzinski et al., 2001; Krieg et al., 1998; Zimmermann et al., 1998). When given with antigens, CpG enhances adaptive immune responses in humans, mice, cattle and sheep, and numerous studies show that CpG is a Th1 promoting adjuvant (Chu et al., 1997; Davis et al., 1998; Ioannou et al., 2003; McCluskie and Davis, 1998).

Much less is known about TLR7 and TLR8 and their ligand interactions. Small anti-viral compounds, imidazoquinolines, were the first agonists for TLR7 and TLR8 to be described (Hemmi et al., 2002). These compounds induce interferons and cytokines in cells from humans and rodents (Hemmi et al., 2002), and protection against HSV, CMV, arbovirus and influenza infections (Chen et al., 1988; Hammerbeck et al., 2007; Herbst-Kralovetz and Pyles, 2006; Kende et al., 1988). Imidazoquinolines also have adjuvant activity in mice and appear to promote Th1 rather than Th2 immune responses (Vasilakos et al., 2000). The natural ligands for TLR7 and TLR8 were recently reported by Heil and colleagues who demonstrated that a sequence from the U5 region of HIV-1 RNA, which is single stranded and rich in guanosine and uracil-activated mouse dendritic cells (DC) to secrete TNF-α and IL-12p40, and human peripheral blood mononuclear cells (PBMCs) to secrete IFN-α (Heil et al., 2004). Also, other investigators found that genomic ssRNA from influenza virus induced production of IFN-α in mouse dendritic cells (Diebold et al., 2004). Cells from mice deficient in TLR7 (TLR7$^{-/-}$) were unresponsive to viral ssRNA, implying that TLR7 is the receptor that detects ssRNA in mice (Heil et al., 2004). However, mice lacking TLR8 responded normally to viral ssRNA. In contrast, human embryonic kidney cells (HEK) transfected with human TLR8 strongly induced NFkB activation upon stimulation with ssRNA (Heil et al., 2004), suggesting species differences among the TLRs (Heil et al., 2004). Evidence from other studies in mice indicated that the ability of vesicular stomatitis virus to stimulate IFN-α secretion in vivo depended on the functional expression of TLR7 and MyD88 (Lund et al., 2004). Therefore, it appears that mouse and human TLR7 and human TLR8 detect certain viral ssRNA.

However, whether TLR7/8 are functional or whether viral or synthetic ssRNA can activate immune cells from domestic animals has not been investigated. Here, we report that the known TLR7/8 ligands, as well as imidazoquinolines, activate immune cells from cattle to produce a variety of cytokines and that CD14^{+} cells are required for these responses.

2. Materials and methods

2.1. Oligoribonucleotides and imidazoquinolines

The ORN used in these studies (designated R-1075) was obtained from Coley Pharmaceutical Group (Wellesley, MA, USA) and this ORN is derived from an HIV sequence previously shown to activate TLR7-expressing cells from mice (Heil et al., 2004). The sequence for this ORN is: 5'-CCGUCUGUUGUGUGACU-3'. A-class CpG ODN 8954; sequence GGGGACGACGTCGTGGGGGGG and C-class CpG ODN 2429; sequence TCGTCGTTTTCGGCGGCCGCCG were provided by Coley Pharmaceutical. The ORN and ODN were all synthesized under endotoxin-free conditions and purified by HPLC. The imidazoquinolines imiquimod and gardiquimod used in these studies were purchased from InvivoGen (San Diego, CA, USA) and were known to be endotoxin-free. Other reagents included LPS (Sigma–Aldrich, Ontario, Canada), DOTAP (Roche Applied Science, Indiana, USA) and bafilomycin (Tocris Bioscience, Missouri, USA).

2.2. Animals

Adult angus–hereford cross cattle of either sex were obtained from the Department of Poultry and Animal Science (University of Saskatchewan, Saskatoon, SK, Canada). The animals were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility and fed ad libitum. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

2.3. Cell isolation and culture

Blood was collected from the jugular vein of cattle by venipuncture using 50 ml syringe containing 2 ml of 7.5% EDTA. The blood was centrifuged at 1400 × g for 20 min and the white blood cell-containing buffy coat was removed and re-suspended in phosphate-buffered saline (PBS; 10 mM, pH 7.4) containing 0.1% EDTA. Peripheral blood mononuclear cells were obtained by overlaying the buffy coat on 54% Percoll™ (Pharmacia Biotech AB, Uppsala, Sweden) and centrifugation at 2000 × g for 20 min. The PBMC were then subjected to three washes using PBS (containing 0.1% EDTA). First, the cells were washed by spinning at 350 × g for 8 min. The pellet was then re-suspended and washed twice by spinning at 150 × g for 8 min to deplete platelets. Stimulation of PBMC was performed in 96-well, round bottom plates (Nunc, Naperville, IL, USA). The PBMC were re-suspended in AIM-V medium supplemented with 2% fetal bovine serum (FBS), 50 μg/ml streptomycin sulphate, 10 μg/ml gentamycin

sulphate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, all from Sigma–Aldrich. For each treatment, 5×10^5 cells were cultured in triplicate wells in 200 μl total volume. Cells were incubated for up to 48 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity.

2.4. Stimulation of cells with RNA ORN

To determine the concentration of ORN for optimal stimulation of bovine cells, we stimulated PBMC with fourfold dilutions of ORN ranging from 10 to 0.391 μg/ml for 48 h and measured IL-12 and IFN-γ concentrations in cell culture supernatants. The ORN was used in the presence of DOTAP at ORN:DOTAP ratio of 1:2. The 48 h time point was previously shown to be optimal time for detection of cytokine responses in bovine PBMC stimulated with a variety of TLR ligands (Mena et al., 2003a). We therefore used 48 h incubation for all cell stimulation experiments. It was previously established using murine and human cells that the immunostimulatory action of ORN requires the presence of 1,2-dioleoyl-3-trimethylam-monium-propane (DOTAP), which enhances the stability and uptake of RNA by cells (Heil et al., 2004). To test whether the presence of DOTAP has any effect on the immunostimulatory activity of ORN on bovine cells, we stimulated PBMC with ORN in the presence (ORN + DO-TAP) or absence of DOTAP (ORN alone) and measured the cytokine responses by ELISA. In this experiment and all other subsequent experiments, ORN was used at 2.5 μg/ml and DOTAP (Sigma–Aldrich) at 5 μg/ml (ORN:DOTAP ratio 1:2). Cells incubated with media alone, A-class CpG 8954 (10 μg/ml), C-class CpG ODN 2429 (10 μg/ml), DOTAP alone, or lipopolysaccharide (LPS; 100 ng/ml) were also included as controls. To compare the immunostimulatory activity of ORN with that of known TLR7/8 ligands, we stimulated PBMC with ORN + DOTAP and two known TLR7/8 ligands; imiquimod (10 μg/ml) and gardiquimod (5 μg/ml) all from Invivogen (San Diego, California). We also explored the kinetics of cytokines induced by ORN in cattle. PBMC were stimulated with ORN + DOTAP and culture supernatants harvested after 6, 24 and 48 h for IFN-α, IFN-γ, IL-12 and TNF-α ELISA.

2.5. ELISA for cytokines

The IFN-α, IFN-γ, IL-12 and TNF-α ELISA used during this study were previously shown to detect bovine and ovine IFN-γ (Mutwiri et al., 2000), IFN-α (Hughes et al., 1994), TNF-α (Ellis et al., 1993) and IL-12 (Hope et al., 2002). Briefly, polystyrene microtitre plates (Immulon 2; Dynex Technology Inc., Chantilly, USA) were coated with capture antibody in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), at 4 °C for 16 h. For the IFN-γ ELISA, mouse anti-bovine IFN-γ antibody (clone 2-2-1A, VIDO) was diluted to 1:8000; for IFN-α ELISA, two mouse anti-bovine IFN-α antibodies (clones IFN-A2 and IFN-A4) were both diluted to 1:1000 and for TNF-α, mouse anti-bovine TNF-α antibody (clone 1D11-13, VIDO) was diluted at 1:1000. Plates were washed with TBST (tris-buffered saline tween buffer; 10 mM Tris–HCl, pH 7.4, 150 mM sodium chloride, 0.05% Tween 20). Ten serial

twofold dilutions of recombinant bovine IFN-γ, bovine IFN-α and bovine TNF-α (Ciba Geigy), starting at 2 ng/ml were used as standards. The diluent for the standards, samples and detection antibodies was TBST containing 0.1% gelatine (Sigma–Aldrich). To detect bound cytokine, rabbit anti-bovine IFN-γ antisera (1:5000), rabbit anti-bovine IFN-α antisera (1:4000), and rabbit anti-bovine TNF-α antisera (Pool 88) (1:1500) were added. Biotiny-lated goat anti-rabbit IgG (1:10,000) (Zymed, South San Fransisco, CA, USA) and streptavidin–alkaline phosphatase (1:10,000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were used for detection. The assay was developed by using 10 mg/ml p-nitrophenyl phos-phate (pNPP) substrate (Sigma–Aldrich) in 1% diethano-lamine (Sigma–Aldrich) and 0.5 mg/ml magnesium chloride. The reaction was stopped by adding 30 μl of 0.3 M EDTA to each well. Optical density of the reaction product was measured at 405 nm using a 490 nm reference on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Sample concentrations were calculated using Microplate Manager 5.0.1 version software (Bio-Rad). For the IL-12 assay (Hope et al., 2002) microtitre plates (Maxisorp, Nunc, Denmark) were coated with mouse anti-recombinant bovine IL-12 antibodies (MCA 1782Z, Serotec, NC, USA) diluted to 8 μg/ml in coating buffer. After washing with TBST, the plates were blocked with TBS (tris-buffered saline; pH 7.4, 0.05 mM) containing 0.1% casein (Sigma–Aldrich) for 1 h at room temperature. Using the blocking buffer as diluent for the remaining steps, recombinant human IL-12 (Serotec PHP 100) was used as the standard starting from 2000 ng/ml. To detect bound cytokine, biotinylated mouse anti-bovine IL-12 (Serotec MCA 2173B) was applied followed by streptavi-din–alkaline phosphatase and pNPP as above.

2.6. CD14 cell enrichment and depletion

PBMC from three animals were fractionated into two highly enriched CD14+ and depleted CD14− fractions by magnetic-activated cell sorting (MACS). Briefly, PBMC were re-suspended in a buffer; PBS (containing 0.5% BSA and 2 mM EDTA), at a concentration of 10^7 cells per 80 μl buffer and labelled with 20 μl mouse anti-human CD14 MACS beads (Miltenyi Biotech GmbH, Germany) per 10^7 cells. After 15 min incubation at 4 °C, the cells were washed by centrifugation at $325 \times g$ for 8 min. The cells were then applied on a LS MACS column, placed in a MACS separator and the effluent containing the unlabelled CD14− cells collected. The column was washed three times using 3 ml of buffer and finally removed from the separator. The CD14+ cells were collected by flushing 5 ml of buffer through the column using the plunger. For additional enrichment/depletion, the CD14+ and CD14− fractions were further passed through separate freshly prepared columns and collected as already described. The purity of CD14+ and CD14− fractions was determined by flow cytometry.

2.7. Flow cytometry

Samples from the un-fractionated PBMC, the enriched (CD14+) and depleted (CD14−) cells were stained for CD14

using the IgG1 monoclonal mouse anti-bovine CD14 antibody (MM61A, VRMD, Pullman, WA, USA). Briefly, the cells were re-suspended at 10^7 per ml in the flow cytometry buffer (PBS; 0.03% sodium azide; 0.2% gelatine). The cells were added to wells of U-bottomed 96-well plates at 100 μl per well and stained with 100 μl of 1:200 diluted mouse anti-bovine CD14 Mab. After 15 min incubation at 4 °C, the cells were washed three times by adding 200 μl of the flow cytometry buffer and centrifugation of plates at $349 \times g$ for 2 min. The cells were then counterstained with FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL, USA), incubated for 15 min and washed three times as already described. Appropriate controls including unstained cells and cells stained with isotype control (IgG1) were also included. Flow cytometry analysis was performed on FACSCalibur® flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA).

2.8. ELISPOT assay for IFN-γ and IL-12

ELISPOT plates (Unifilter 350, Whatman, NJ, USA) were coated with capture antibodies at 4 μg/ml mouse anti-bovine IL-12p40 (MCA 1782EL, ABD Serotec, Oxford, UK) or 0.125 μg/ml mouse anti-recombinant bovine IFN-γ (clone 2-2-1A) and incubated overnight at 4 °C. The plates were blocked for 1 h with AIM-V media containing 10% FBS and washed three times with sterile PBS. The cells were added in triplicate wells as follows: for un-fractionated PBMC, 5×10^5 cells were added to each well. However, for enriched (CD14$^+$) and the depleted (CD14$^-$) fractions, the number of cells added to each well depended on the proportion (%) of these cells in PBMC as pre-determined by flow cytometry. For example, for an animal whose PBMC profile showed 15% CD14$^+$ and 85% CD14$^-$, the number of cells added to each well was 15% of 5×10^5 cells for CD14$^+$ and 85% of 5×10^5 cells for the CD14$^-$ fraction. To make the "reconstituted CD14$^+$/CD14$^-$", the two fractions were added-back together based on their proportion in the respective animal PBMC. The various cell populations (un-fractionated PBMC, CD14$^+$, CD14$^-$, reconstituted cells) were stimulated with ORN delivered with DOTAP at final concentrations of 2.5 and 5 μg/ml, respectively, and incubated for 12 h at 37 °C, 5% CO_2 in a humidified incubator. Un-stimulated control cells were kept for each cell type in triplicate wells in AIM-V media. From this stage, the spots that represent cytokine secreting cells, were developed by addition of reagents at each step followed by 2 h incubation at room temperature and finally washing the plates six times with PBST (PBS containing 0.05% Tween 20). Interferon-γ spots were detected indirectly though addition of the rabbit anti-bovine IFN-γ polyclonal antisera (lot 92-131) followed by addition of the biotinylated goat anti-rabbit mab (Zymed, South San Fransisco, CA, USA). Interleukin-12 spots were detected directly by addition of the biotinylated mouse anti-bovine IL-12 monoclonal antibody (MCA 2173B, Serotec). The spots were visualized by addition of streptavidin–alkaline phosphatase (ImmunoResearch Laboratories, West Grove, PA, USA) and NBT/BCIP (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The spots were counted under an inverted microscope and recorded as number of cytokine secreting cells.

2.9. RNA extraction and quantitative real-time PCR

Three cell populations including the un-fractionated PBMC, the CD14-depleted PBMC and the CD14$^+$ cells were pelleted at $300 \times g$ for 5 min at 4 °C immediately after purification. These cells were lysed with 1 ml Trizol (Invitrogen) and collected in a 1.5 ml eppendorf tube and total RNA extraction was performed as described previously (Aich et al., 2005). RNA amplification was performed using the MessageAmp™ II aRNA Amplification Kit (Applied Biosystems/Ambion, Inc., Austin, TX) as per manufacturer's instructions. Expression of mRNA for TLR7 and TLR8 in the three cell populations was quantified using quantitative real-time PCR (qRT-PCR) exactly as described (Menzies and Ingham, 2006). Analysis was performed in duplicate using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen) on the Bio-Rad iCycler (Bio-Rad Laboratories) as per kit manufacturer's instructions. Expression of GAPDH was used to verify that the quantity of starting material was equivalent across each template. Melt curve analysis was performed to ensure that any product detected by the iCycler was specific to the desired amplicon. We used the primers previously reported (Menzies and Ingham, 2006) and the sequences are shown in Table 1. Quantitative RT-PCR data were analysed with the comparative C_T method ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001). The difference (ΔC_T) between the C_T values of the target and the normalizer (GAPDH), i.e. ($\Delta C_T = C_T$ [target] $- C_T$ [GAPDH]) was calculated for the three cell subpopulations. Using the CD14-depleted subpopulation as the reference, we calculated the $\Delta\Delta C_T$ by calculating the difference between the sample ΔC_T and the reference ΔC_T. The $\Delta\Delta C_T$ obtained were subsequently transformed to absolute values using the formula: comparative expression level = $2^{-\Delta\Delta C_T}$.

2.10. Statistical analysis

Data involving more than two samples were analysed by one-way variance (ANOVA) while data from time course experiments was analysed by two-way ANOVA using GraphPad Prism 5 (GraphPad Software, Inc., CA). Data that were not normally distributed were logarithmically transformed and the means were compared by the Turkey's test. Three levels of significance were detected and designated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. RNA ORN induce IL-12 and IFN-γ in a dose-dependent manner

We conducted a fourfold dose titration to determine the optimal concentration of ORN + DOTAP (ORN:DOTAP ratio 1:2) required for cytokine induction. Results show that ORN induced IL-12 and IFN-γ in a dose-dependent manner. Compared to un-stimulated cells (media control), ORN

Table 1
Sequence of primers used in quantitative real-time PCR

TLR	Sequence	Size (bp)	Efficiency
TLR7	Forward: ACTCCTTGGGGCTAGATGGT; reverse: GCTGGAGAGATGCCTGCTAT	180	1.92
TLR8	Forward: TCCACATCCCAGACTTTCTACGA; reverse: GGTCCCAATCCCTTTCCTCTA	150	1.80
GAPDH	Forward: CCTGGAGAAACCTGCCAAGT; reverse: GCCAAATTCTGTCGTACCA	200	1.93

induced significantly increased IL-12 (Fig. 1A) and IFN-γ (Fig. 1B) starting at 0.156 μg/ml ($p < 0.01$) and 0.625 μg/ml, respectively. Further increase in ORN dose caused significant ($p < 0.001$) increase in IL-12 and IFN-γ responses that peaked at 0.625 and 2.5 μg/ml, respectively (Fig. 1A and B). Therefore we selected ORN dose of 2.5 μg/ml for all subsequent cell stimulation studies.

3.2. The stimulatory activity of ORN requires the presence of DOTAP

ORN used in these studies was delivered with DOTAP which is thought to enhance the stability and uptake of RNA by cells (Heil et al., 2004). To rule out the possibility that DOTAP was responsible for the cytokine responses, PBMC were incubated with ORN alone, DOTAP alone or ORN + DOTAP. As expected, ORN + DOTAP induced significantly ($p < 0.001$) high levels of IL-12 (Fig. 2A), TNF-α (Fig. 2B) and IFN-γ (Fig. 2C) responses. However, ORN + DOTAP failed to induce any detectable IFN-α responses (Fig. 2D). The IL-12, TNF-α and IFN-γ responses induced ORN alone or DOTAP alone were significantly lower ($p < 0.001$) to those induced by ORN + DOTAP (Fig. 2A–C). CpG ODN is known to stimulate cytokine production in immune cells from cattle (Mena et al., 2003b). For this reason, we used A-class CpG 8954 and C-class CpG2429 as controls. Significant IL-12 responses were induced by both A-class CpG8954 ($p < 0.001$) and C-class CpG 2429 ($p < 0.001$) as well as LPS ($p < 0.01$) (Fig. 2A). Interestingly, ORN + DOTAP induced a higher IL-12 ($p < 0.05$) and TNF-α ($p < 0.01$) and IFN-γ ($p < 0.001$) responses than A-class CpG8954, C-class CpG ODN 2429 or

LPS (Fig. 2A–C). Our results show qualitative similarities and differences in cytokine responses induced by ORN and CpG (A- and C-class). While both ORN + DOTAP and A- and C-class CpG induced significant ($p < 0.001$) IL-12 responses (Fig. 2A), ORN and CpG were different in that ORN + DOTAP did not induce IFN-α (Fig. 2D) while A- and C-class CpG failed to induce TNF-α or IFN-γ (Fig. 2B and C). We then compared ORN with other known TLR7/8 ligands, imiquimod and gardiquimod. All the three TLR7/8 ligands induced significant ($p < 0.001$) IL-12 and IFN-γ secreting cells (Fig. 3). The most potent inducer of IL-12 secreting cells was gardiquimod followed by imiquimod and lastly ORN + DOTAP. Imiquimod and ORN + DOTAP induced similar numbers of IFN-γ secreting cells which however were significantly lower ($p < 0.01$) to those induced by gardiquimod.

3.3. The kinetics of IL-12, TNF-α and IFN-γ induced by ORN are different

ORN + DOTAP induced production of IL-12 as early as 6 h ($p < 0.01$) after stimulation and the response increased significantly ($p < 0.001$) to peak levels at 24 and 48 h (Fig. 4A). The ORN + DOTAP-induced TNF-α response on the other hand increased dramatically ($p < 0.001$) and was at peak levels as early as 6 h and the levels were maintained through 24–48 h (Fig. 4B). The IFN-γ response however was delayed in that it was undetectable at 6 h but increased significantly ($p < 0.001$) to reach peak levels at 24 h through 48 h (Fig. 4C). Responses induced by DOTAP alone or ORN alone were not different from un-stimulated cells (media).
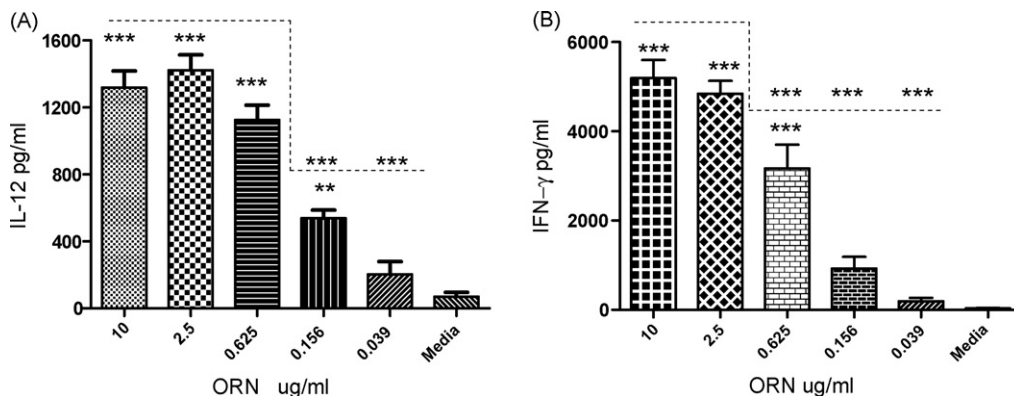

Fig. 1. RNA oligoribonucleotides (ORN) induces IL-12 (A) and IFN-γ (B) in a dose-dependent manner. Peripheral blood mononuclear cells from cattle were stimulated with fourfold dilutions of ORN ranging from 10 to 0.039 μg/ml for 48 h and the concentration of IL-12 and IFN-γ in culture supernatants measured by ELISA. Data represents mean ± S.D. of five animals. Differences between stimulated and un-stimulated cells or between ORN doses were analysed using one-way ANOVA. The * sign immediately above the bar shows difference relative to un-stimulated cells while the * sign above the dotted line shows difference relative to the highest response (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
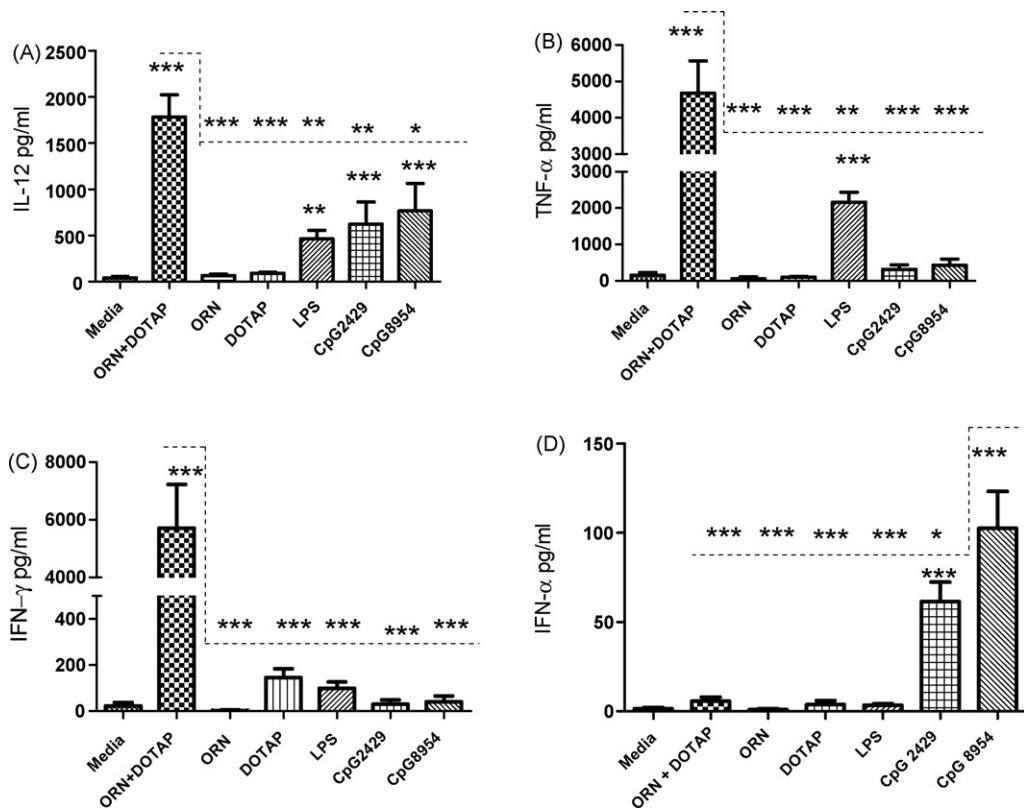
Fig. 2. RNA oligoribonucleotides (ORN) require DOTAP to induce IL-12, TNF-α and IFN-γ responses. Peripheral blood mononuclear cells from cattle were stimulated for 48 h with 2.5 µg/ml of ORN (R-1075) alone or linked with DOTAP (5 µg/ml). For control purposes, PBMC were also stimulated with media, 2.5 µg/ml ORN alone, 5 µg/ml DOTAP alone, 10 µg/ml A-class CpG 8954, 10 µg/ml C-class CpG 2429 or LPS (100 ng/ml). The concentration of IL-12 and TNF-α, IFN-γ and IFN-α in culture supernatants was measured by ELISA. Data represents mean ± S.D. of five animals and was analysed by one-way ANOVA. Differences in cytokine responses between stimulated and un-stimulated (media) is shown by * above the bars. The * above the dotted line shows statistical difference relative to the highest response (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
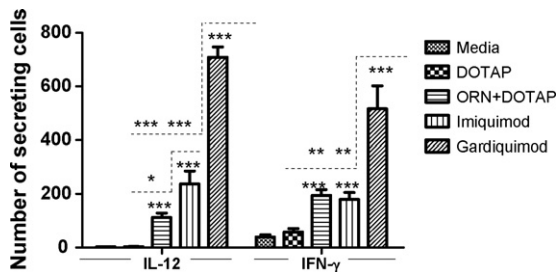

Fig. 3. Comparison of the immunostimulatory activity of RNA oligoribonucleotides (ORN) with other known TLR7/8 ligands. Peripheral blood mononuclear cells from cattle were stimulated for 48 h with ORN (2.5 µg/ml) or other known TLR7/8 ligands including imiquimod (10 µg/ml) and gardiquimod (5 µg/ml). The number of IL-12 or IFN-γ secreting cells was measured by the ELISPOT assay. Data represents mean ± S.D. of five animals and expressed as IL-12 secreting cells per 2.5×10^5 cells or IFN-γ secreting cells per 4×10^5 cells. Data was analysed using one-way ANOVA. Statistical difference in responses between un-stimulated and cells stimulated with different stimulants is shown by * above the bars. The * above dotted line shows statistical differences relative to the highest response (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.4. Induction of cytokines by ORN requires engagement of an intracellular receptor

Successful signalling through intracellular TLRs requires acidification and maturation of phagosomes. To determine whether interference with this process leads to inhibition of ORN induction of cytokine responses in bovine PBMC, we pre-treated cells with bafilomycin (100 nM), a V-type ATPase inhibitor, prior to stimulation of cells with ORN. Control wells included cells treated with LPS, which signals through an extracellular receptor, TLR4. Pre-treatment of cells with bafilomycin significantly reduced the TNF-α response ($p < 0.001$) following stimulation with ORN + DOTAP (Fig. 5). However, bafilomycin treatment had no effect on the TNF-α response to LPS stimulation. These results suggest that ORN signalling requires endosomal maturation and suggests that the receptor is intracellular.

3.5. CD14+ cells are required for ORN-induced IL-12 and IFN-γ PBMC responses

Flow cytometry results showed that CD14+ and CD14− cells represented $16.77 \pm 0.38\%$ and $83.22 \pm 0.38\%$ of PBMC,
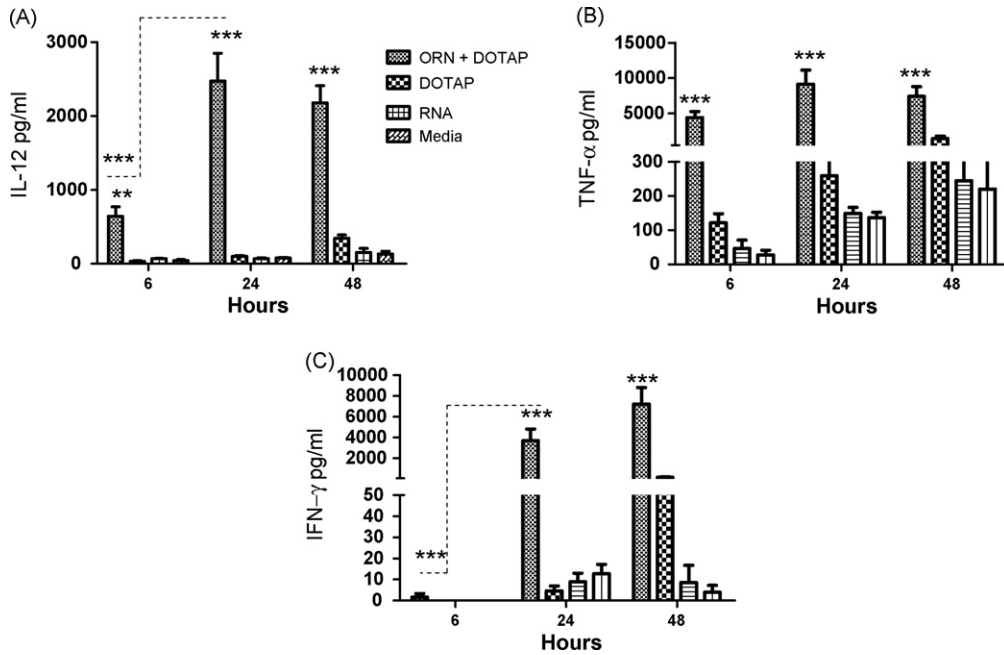
Fig. 4. Kinetics of IL-12, TNF-α and IFN-γ responses following stimulation with RNA oligoribonucleotides (ORN). Peripheral blood mononuclear cells from cattle were stimulated with ORN (2.5 μg/ml) for up to 48 h and the cytokine responses quantified by ELISA. Data represents mean $\pm$ S.D. of five animals and was analysed by two-way ANOVA. Differences in cytokine responses between stimulated and un-stimulated (media) cells is shown by * above the bars. Star sign above the dotted line shows differences relative to the peak response ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

respectively. The purity of the CD14-enriched (CD14+) cells was $96.81 \pm 0.62\%$ while that of the CD14-depleted (CD14−) cells was $97.48 \pm 0.99\%$ (data not shown). Depletion of CD14+ cells from PBMC resulted in near complete loss of IL-12 secretion in the CD14− fraction (Fig. 6). However, the CD14+ fraction retained significant ($p < 0.05$) IL-12-secreting capacity though significantly ($p < 0.001$) reduced when compared

to reconstituted CD14+/CD14− or PBMC (Fig. 6). These results show that the CD14+ fraction alone responds directly to ORN + DOTAP, by producing suboptimal IL-12 but full response is restored in the presence of both the CD14− and the CD14+ cells. On the other hand, ORN + DOTAP only induced IFN-γ secretion in the presence of both the CD14+ and the CD14− fractions since the responses by the separated
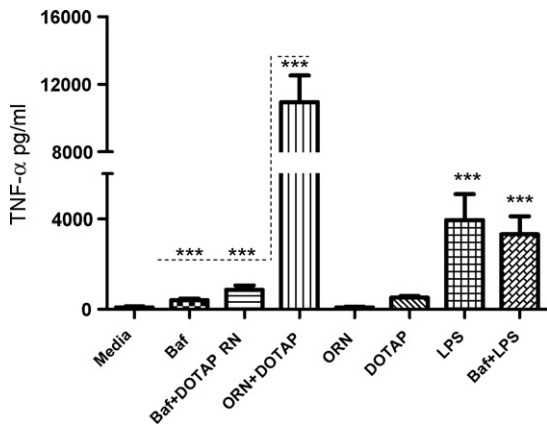

Fig. 5. Inhibition of phagosomal maturation by bafilomycin (Baf) interferes with the immunostimulation activity of RNA oligoribonucleotides (ORN) but not LPS. Peripheral blood mononuclear cells from cattle were stimulated for 48 h with ORN (2.5 μg/ml) or LPS (100 ng/ml) in the presence or absence of bafilomycin (100 nM). TNF-α was measured by ELISA. Data shown represents mean $\pm$ S.D. of five animals and was analysed using one-way ANOVA. Differences between stimulated and un-stimulated cells is shown by * directly above the bars. The * above the dotted line shows differences relative to the highest response ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).
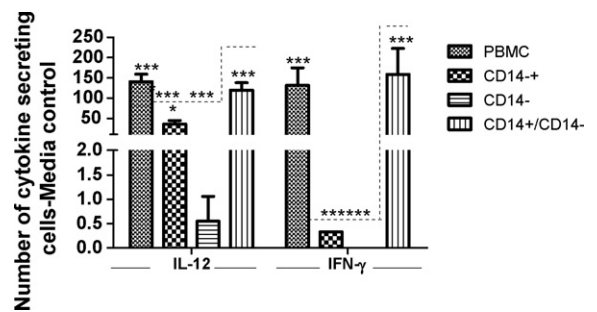

Fig. 6. The CD14+ cells are required for optimal IL-12 and IFN-γ induction by RNA oligoribonucleotides (ORN). PBMC were fractionated into CD14+ and CD14− cell populations using magnetic-activated cell sorting (MACS). The un-fractionated PBMC, the CD14+, CD14− and the reconstituted (CD14−/CD14+) were stimulated with ORN (R-1075) (2.5 μg/ml) for 18 h and the number of IL-12 or IFN-γ secreting cells quantified using the ELISPOT assay. The average number of cells per well of 97-well plate were 5×10^5 for PBMC [(16.77%) $\times$ (5 $\times$ 10^5)] for CD14+ cells, and [83.23% $\times$ (5 $\times$ 10^5)] for CD14− cells. Data represent mean $\pm$ S.D. less than the background (cytokine secreting cells in un-stimulated cells). Significant differences between stimulated and un-stimulated cells is shown by * directly above the bars while the * above the dotted line shows differences relative to the highest response ($^*p < 0.05$, $^{**}p < 0.01$, $p < 0.001$).
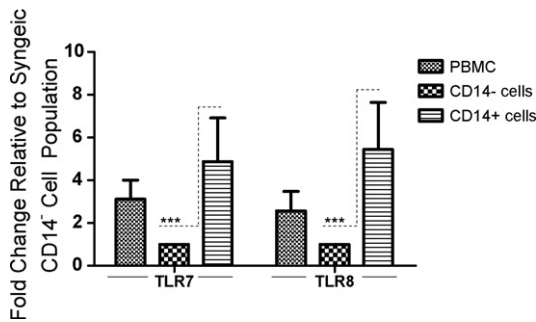
Fig. 7. TLR7 and TLR8 mRNA expression by purified CD14^{+}, CD14^{−} and un-fractionated bovine peripheral blood mononuclear cells (PBMCs). PBMC was fractionated into CD14^{+} and CD14^{−} fractions using magnetic-activated cell sorting. Expression of TLR7 and TLR8 mRNA in the CD14^{+}, CD14^{−} and un-fractionated PBMC was quantified using quantitative real-time PCR (RT-PCR).

fractions (CD14^{+} or CD14^{−} cells) was significantly lower ($p < 0.001$) to undetectable (Fig. 6).

3.6. CD14^{+} peripheral blood mononuclear cells express both TLR7 and TLR8

We quantified the TLR7 and TLR8 mRNA expression in the three cell populations: un-fractionated PBMC, CD14^{+} cells and the CD14^{+} cells. All the three populations expressed both TLR7 and TLR8 (Fig. 7). However, expression of TLR7 and TLR8 was significantly higher ($p < 0.001$) in the CD14^{+} fraction than the CD14^{−} PBMC fraction.

4. Discussion

The present investigations reveal for the first time that known TLR7/8 ligands are potent activators of immune cells from ruminants and that optimum induction of IL-12 and IFN-γ by these ligands requires the presence of both the CD14^{+} and CD14^{−} PBMC subpopulations. These findings strongly warrant studies on possible role of the TLR7/8 ligands as vaccine adjuvants in cattle.

Our results reveal differences in potency of the TLR7/8 ligands tested as well as qualitative differences in the cytokine responses induced by ORN and CpG, the TLR9 ligand. Of the three TLR7/8 ligands tested, gardiquimod was the most potent inducer of IL-12 followed by imiquimod. ORN and imiquimod induced similar levels of IFN-γ but these were significantly lower than those induced by gardiquimod. These results confirm previous observations that gardiquimod is more potent than imiquimod (Stamatatos et al., 1988). Compared to CpG, ORN was a potent stimulator of IFN-γ and TNF-α but not IFN-α while CpG had an opposite effect. These observations are in sharp contrast to what has been described in humans. pDC from humans express both TLR7 and TLR9 (Hornung et al., 2002), and have been identified as the primary source of type I interferons in PBMC stimulated with ssRNA or ORN and CpG, respectively (Diebold et al., 2004; Ishii and Akira, 2006). In cattle, putative pDC have been described, and similar to the situation in humans, these cells are thought to produce IFN-α in bovine PBMC stimulated with CpG DNA (Griebel et al., 2005) suggesting

that bovine pDC express TLR9. The lack of IFN-α production in bovine PBMC stimulated with ORN is perplexing since our results show that these cells express TLR7 and TLR8. Other factors such as differences in the TLR7/8/9 signalling pathways between human and bovine may account for the discrepancy.

ORN activated IL-12, TNF-α and IFN-γ only in the presence of DOTAP similar to previous observations (Boczkowski et al., 1996; Heil et al., 2004). DOTAP is a cationic lipid which is used to facilitate intracellular delivery of macromolecules such as plasmid, DNA or RNA through a mechanism that is not well understood. It has been suggested that DOTAP forms electrostatic association, coats and condense the macroparticles to a form that is suitable for cellular uptake (Hafez et al., 2001). Furthermore, the positive charge on DOTAP is suggested to facilitate the association with the negatively charged cell surface leading to uptake by endocytosis (Stamatatos et al., 1988). DOTAP is also thought to increase the retention of ORN in endosomes which helps to increase duration of ORN-TLR engagement (prolong activation) before ORN is eventually digested by lysosomal enzymes (Hafez et al., 2001). Our results supports previous findings that the ORN receptor(s) are localized intracellular (in endosomes) and activation requires maturation of endosomes since the immune stimulation activity of ORN was abrogated in the presence of bafilomycin which interferes with endosomal maturation (Heil et al., 2003). In contrast, the immunostimulation activity of LPS was not blocked by bafilomycin because the respective receptor TLR4 is localized on cell surface.

Although signalling by TLR7/8 ligands can potentially induce both type 1 interferons and proinflammatory cytokines (Gorden et al., 2005; Ishii and Akira, 2005; Kawai and Akira, 2007), studies using human PBMC shows that while TLR7 selective ligands induces a predominantly type 1 interferon response, the TLR8 selective ligands predominantly induces proinflammatory cytokines and chemokines (Ghosh et al., 2006; Krieg, 2006). In the present study using PBMC from cattle, the TLR7/8 ligand ORN induced a strong proinflammatory cytokine response (IL-12, TNF-α and IFN-γ) but failed to induce IFN-α (type 1 interferon). This cytokine pattern is strongly suggestive of TLR8 activation rather than TLR7 and that ORN R-1075 may be selective for TLR8. However, no studies have been done in cells from cattle to determine whether selective agonists for TLR7 or TLR8 elicit a cytokine pattern similar to that seen in humans. Such studies are needed in order to fully understand the immune modulatory activities of TLR7/8 ligands in cattle.

By monitoring the IL-12 and IFN-γ secreting cells, we revealed cellular cooperation between the CD14^{+} and the CD14^{−} subpopulations in the innate immune responses to ORN. Isolated CD14^{+} cells responded directly to ORN by secreting IL-12, though suboptimal levels, while isolated CD14^{−} cells did not respond with IL-12 or IFN-γ. Based on the reported expression of CD14 in cattle, it follows that fractionation of PBMC separated the IL-12-producing DC/monocytes into the CD14^{+} subpopulation and the IFN-γ-producing NK cells into the CD14^{−} subpopulation (Pinchuk et al., 2003) and this fits well with the cytokine pattern

observed in other studies (Hsieh et al., 1993; Trinchieri, 1997, 2003; Unanue, 1997). We hypothesize that because DC/monocytes (CD14$^+$) express high levels of TLR7/8, they responded directly to ORN with IL-12 response but could not produce IFN-γ because the IFN-γ-producing cells (NK cells) were depleted. The NK cells (CD14$^-$) failed to respond directly to ORN because they either do not or express low levels of TLR7/8 and are not directly activated by ORN through the DC/monocytes (CD14$^+$) or their products including IL-12. This is consistent with the comparatively higher TLR7/8 expression in the CD14$^+$ than the CD14$^-$ subpopulations. Alternatively, the NK cells (CD14$^-$) express TLR7/8 but require additional signals from the DC/monocytes as has been described for human NK cells (Girart et al., 2007; Hart et al., 2005). Furthermore, we suggest that isolated CD14$^+$ cells secreted suboptimal levels of IL-12 because they require additional signals from CD14$^-$ cells for optimal responses in view of the cross-talk or cross-regulation between the DC and NK cells as previously described (Marcenaro et al., 2006; Moretta, 2005; Pan et al., 2004; Zitvogel et al., 2006). This cross-regulation involves direct contact through formation of stimulatory synapses and indirect contact through secreted cytokines leading to DC-induced NK-cell proliferation, NK-cell-mediated cytokine release and NK-cell-dependent DC maturation.

In summary, we have established that RNA oligoribonucleotides, the natural ligands for TLR7/8 are potent innate immune stimulators in cattle and this warrants further studies on their use as vaccine adjuvants.

Acknowledgements

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