Bovine Plasmacytoid Dendritic Cells Are the Major Source of Type I Interferon in Response to Foot-and-Mouth Disease Virus *In Vitro* and *In Vivo*[▽]

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Type I interferons (alpha/beta interferons [IFN- α/β]) are the main innate cytokines that are able to induce a cellular antiviral state, thereby limiting viral replication and disease pathology. Plasmacytoid dendritic cells (pDCs) play a crucial role in the control of viral infections, especially in response to viruses that have evolved mechanisms to block the type I IFN signal transduction pathway. Using density gradient separation and cell sorting, we have highly enriched a population of bovine cells capable of producing high levels of biologically active type I IFN. These cells represented less than 0.1% of the total lymphocyte population in blood, pseudoafferent lymph, and lymph nodes. Phenotypic analysis identified these cells as bovine pDCs (CD3⁺ CD14⁺ CD21⁺ CD11c⁺ NK⁺ TCR δ ⁺ CD4⁺ MHC II⁺ CD45RB⁺ CD172a⁺ CD32⁺). High levels of type I IFN were generated by these cells *in vitro* in response to Toll-like receptor 9 (TLR-9) agonist CpG and foot-and-mouth disease virus (FMDV) immune complexes. In contrast, immune complexes formed with UV-inactivated FMDV or FMDV empty capsids failed to elicit a type I IFN response. Depletion of CD4 cells *in vivo* resulted in levels of type I IFN in serum early during FMDV infection that were significantly lower than those for control animals. In conclusion, pDCs interacting with immune-complexed virus are the major source of type I interferon production during acute FMDV infection in cattle.

Foot-and-mouth disease virus (FMDV) is the etiological agent responsible for foot-and-mouth disease, an economically important, highly contagious, acute vesicular disease affecting cloven-hoofed animals, including domesticated animals such as cattle, sheep, pigs, goats, camelids, and deer. FMDV is a member of the *Aphthovirus* genus in the family *Picornaviridae*. Seven immunologically distinct FMDV serotypes have been identified (O, A, C, SAT1, SAT2, SAT3, and Asia1), and within each serotype a large number of strains have evolved due to the high rates of genomic mutation that are common in RNA viruses (26, 45). FMDV has a single positive-stranded RNA genome of 7.5 to 8.5 kb and is attached to a VPg protein that acts as a primer for viral RNA replication. The genome is polyadenylated and encodes a single polypeptide that is processed in infected cells to yield polypeptide precursors and mature viral proteins. More than 50% of ruminants exposed to viral challenge, whether vaccinated or not, can become carriers (2). A carrier of FMDV is defined as an animal from the scrapings of whose oropharynx live virus can be recovered after 28 days following infection (2). Curiously, the carrier state has not been reported in pigs (71).

FMDV has been shown to antagonize the host innate response in mammalian cells by inhibiting the induction of antiviral molecules at both the translational level (35) and the transcriptional level (20, 65), interfering with the secretory pathway (51, 52) and inhibiting surface expression of viral proteins (56). A key component of this antagonism is orches-

trated by the FMDV Leader proteinase (L^{pro}), which targets the host translation initiation factor eIF4G for degradation (21, 50) and, as a consequence, blocks *de novo* protein synthesis and the production of type I interferons (alpha/beta interferons [IFNs- α/β]) (21). L^{pro} also disrupts NF- κ B (22) and inhibits double-stranded RNA (dsRNA)-induced type I IFN transcription by orchestrating the degradation of IFN regulatory factor 3/7 (IRF3/7) (65). Mason et al. have shown that a genetically engineered FMDV lacking L^{pro} has an attenuated phenotype *in vitro* and *in vivo* (48). This is attributed to the inability to block the production of type I IFNs, which inhibit FMDV replication (16).

In vitro studies show that FMDV has the capacity to target the innate immune response. Although detailed *in vivo* studies on this subject are lacking, FMDV infection *in vivo* has recently been shown to lead to an increase in the levels of type I IFN in porcine serum (55). Quantitative assays measuring the mRNA expression of IFN- α in nasal mucosa-associated lymphoid tissue (NALT) of cattle infected with FMDV revealed significantly greater levels during the acute disease phase than in uninfected animals (70). In cattle experimentally infected with FMDV, tissue-specific profiles revealed significant increases in IFN- α mRNA levels in the coronary band and tongue at 1 to 2 days postinfection, coinciding with increases in the viral RNA load (69), though it is unclear if this message was translated into protein. Type I IFN has also been detected in the sera of pigs infected with different serotypes of FMDV and has been correlated with the peak of viremia and a transient lymphopenia (55). The observed increase in type I IFN levels during the early stage of FMDV infection is believed to limit viral replication and to contribute to the resolution of viremia prior to the induction of a specific immune response.

Many types of cells are capable of producing type I IFN, but

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the “natural type I IFN-producing cells” (NIPC), also known as plasmacytoid dendritic cells (pDCs), produce very high levels of type I IFN in response to infection with enveloped viruses (31). Nonenveloped viruses induce very little, if any, type I IFN in pDCs (29, 57). However, it has been shown that antibodies complexed to nonenveloped viruses such as FMDV lead to viral uptake, abortive replication, and the production of high levels of type I IFN in porcine pDCs (35). The failure to establish the carrier state in pigs may be related to the production of type I IFN by porcine pDCs in response to FMDV. These cells provide a key link between the innate and adaptive immune responses and are crucial for the control of viral infections, especially for viruses that have the machinery to block IFN induction in target host cells.

pDCs have been isolated and characterized from a number of species, including humans, mice, and pigs (4, 27, 33, 55, 63), but not from cattle. Pascale et al. have identified and partially characterized these cells in sheep (58). We investigated the interaction between FMDV and bovine pDCs in order to determine whether type I IFN was also induced early during FMDV infection in cattle.

In this study we describe (i) the isolation and characterization of bovine pDCs, (ii) the antibody-dependent uptake of virus by these cells, (iii) the induction of type I IFN production in response to virus-immune complexes, and (iv) the reduction of type I IFN levels in sera from infected cattle selectively depleted of specific cell populations.

MATERIALS AND METHODS

Cattle. Calves were conventionally reared British Holstein Friesians bred at the Institute for Animal Health. All experiments were approved through the Institute's ethical review process and were in accordance with national guidelines on animal use.

Cannulation. Cannulations were performed as previously described (37). Lymph was collected into sterile plastic bottles containing heparin (10 U/ml), penicillin, and streptomycin. Bottles were replaced every 8 to 12 h.

Cells and viruses. Seed stocks of FMDV isolate O1K (Kaufbeuren)-Cad2 were a gift from Terry Jackson, Pirbright Laboratory, United Kingdom. O1K-Cad2 was initially propagated in primary bovine thyroid cells (BTY) (Pirbright Laboratory, United Kingdom) to maintain the integrin-binding specificity of the virus. Aliquots of this seed virus were used to infect BHK-21 cells (baby hamster kidney cells strain 21, clone 13; ATCC CL10), obtained from the American Type Culture Collection (Manassas, VA), as described above to produce the working stock of virus.

All cells were maintained at 37°C under 5% CO₂. The MDBK-t/2 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Autogen Bioclear, Wiltshire, United Kingdom), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml blasticidin (32).

Empty capsids. FMDV empty capsids (strain O₁ Manissa) were a kind gift from Alison Burman and were supplied at a concentration of 0.05 µg/ml, which is the equivalent of 6.25×10^9 particles/µl (44). They were used both alone and to form capsid-immune complexes (10^9 particles per reaction).

UV inactivation of FMDV. FMDV (strain O₁K Cad2) was inactivated on ice by UV radiation using an 8-W UV lamp, emitting at 254 nm, placed 10 cm above the virus solution for 15 min. UV-treated virus preparations were checked for inactivation by the World Reference Laboratory Virus Diagnosis Group using their standard protocol (SOP 011-5).

Immune complexes. Antisera from 10 experimentally challenged (strain OUKG 34/2001) animals were used to form immune complexes with O₁K Cad2 virus, UV-inactivated virus, and empty capsids. Sera were diluted (1:10, vol/vol) in virus, and an equivalent volume was used for the empty capsids.

Preparation of lymphoid tissue cell suspensions. Mesenteric lymph nodes (MLN), harvested from naive male animals (3 to 18 months of age), were cut into small pieces and forced through muslin gauze. Released cells were underlaid with Histopaque (density, 1.083 g/ml; Sigma, United Kingdom), centrifuged at $1,200 \times g$ at 18°C for 20 min, and then recovered from the gradient interface.

Isolation of cell suspensions from pseudoafferent lymph and blood. Both pseudoafferent lymph and blood were diluted 1:1 with phosphate-buffered saline (PBS) and were underlaid with Histopaque-1083. Tubes were centrifuged at $1,200 \times g$ and 18°C for 20 min. Cells were collected from the gradient interface and were washed three times in calcium- and magnesium-free PBS.

Iodixanol gradient enrichment of lymphoid tissue cell suspensions for LLD cells. Large low-density (LLD) cell enrichment was performed in line with a previously published protocol used to isolate dendritic cells from mouse splenic lymphocytes (60), with the following changes. Live cells recovered from the Histopaque gradients were resuspended in a 15% iodixanol solution (OptiPrep; Axis-Shield, Kimbolton, United Kingdom) diluted in Hanks balanced salt solution (HBSS) (Sigma, Poole, United Kingdom) and were then overlaid with a 11.5% iodixanol solution diluted in calcium- and magnesium-free PBS and 0.5% bovine serum albumin (BSA). Finally, the gradient was overlaid with 6 ml of HBSS and was centrifuged at $600 \times g$ for 30 min at 18°C (accuSpin model 3R; Fisher Scientific). LLD cells were harvested from the interface and were washed in PBS. LLD cells were maintained in complete growth medium (DMEM, 10% adult bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin).

MACS of large low-density cells using Miltenyi technology. MidiMACS columns were prepared according to the instructions of the manufacturer (Miltenyi Biotec), and 3×10^8 LLD cells were used for each depletion procedure. Monoclonal antibodies (Table 1) were diluted in PBS–0.5% BSA (1 µg/ml for purified antibodies or a 1:10 dilution for tissue culture supernatants) and were incubated with LLD cells for 30 min on ice. LLD cells were washed three times, resuspended in magnetic cell sorting (MACS) column buffer (PBS containing 2% adult bovine serum), incubated with 100 µl of MACS anti-IgG1 beads for 30 min on ice, washed as described above, and resuspended in MACS column buffer. Cells were passed through two MACS LS magnetic columns (Miltenyi Biotec), and the flowthrough was collected. Depleted LLD cells in the flowthrough were resuspended in complete growth medium.

Direct conjugation of antibodies. Zenon Alexa Fluor labeling kits (Invitrogen, United Kingdom) were used according to the manufacturer's protocol to directly label primary antibodies for use in fluorescence-activated cell sorting (FACS) staining procedures.

FACS staining and analysis. Cells were plated at a concentration of 3×10^5 per well in round-bottom 96-well tissue culture plates (Nunc, Roskilde, Denmark). Directly conjugated antibodies (Table 1) diluted in FACS buffer (PBS, 0.5% BSA) were incubated with cells for 20 min on ice, washed with FACS buffer, fixed with 1% paraformaldehyde, and analyzed by FACS using FCS Express software.

Assay for type I IFN production by LLD cells. An Mx promoter-chloramphenicol acetyltransferase (Mx-CAT) reporter assay was used to determine the levels of biologically active type I IFN (32). For each assay, the supernatants from 1×10^6 to 5×10^6 isolated LLD cells or selectively depleted populations of these cells were used. Cells were incubated overnight with FMDV (strain O1K Cad2; multiplicity of infection [MOI], 3) or with immune complexes previously formed using the virus and day 0, day 5, or day 13 bovine antiserum to FMDV (strain OUKG 34/2001) at a 1:10 dilution. Unmethylated CpG oligodeoxynucleotides (2 µM; ODN2216; Invivogen) were used as a positive control for the stimulation of type I IFN (34, 46).

Immunofluorescence and confocal microscopy. Coverslips treated with fetal calf serum (FCS) for 2 h were used to encourage cell adherence. Adherent cells from the depleted LLD population were treated with CpG (final concentration, 2 µM) overnight at 37°C or in the presence of brefeldin A (final concentration, 5 µg/ml) for 4 h at 37°C. Cells were fixed using methanol-acetone fixation or 2% paraformaldehyde, permeabilized using 1% octylphenyl-polyethylene glycol (IGEPAL), blocked with 10% normal goat serum, and incubated with primary antibodies at 1 µg/ml and with secondary (Alexa Fluor) antibodies at a dilution of 1:500 for 30 min on ice. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000).

Western blotting. Large low-density cells and depleted populations (1×10^6 cells per well) were treated as for the IFN production assay. After 24 h of treatment, cells were lysed directly in reducing sample buffer. Western blotting was performed as previously described (25). Primary antibodies, mouse anti- γ -tubulin (T6557; Sigma), and rabbit anti-IRF-7 (Abcam) were all used where indicated. Bound primary antibodies were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Bio-Rad) antibodies.

Statistical analysis. Statistical analysis was carried out using the Minitab 16 statistical analysis program. Levels of type I IFN produced *in vitro* by LLD were log-transformed and analyzed using linear models (analysis of variance [ANOVA]) with tissue and cell source/antigen as fixed effects. Model selection proceeded by stepwise deletion of nonsignificant terms (as judged by *F* tests)

TABLE 1. Mouse monoclonal antibodies used for magnetic cell sorting, fluorescence-activated cell sorting analysis, and immunofluorescence

Antibody nomenclature	Antigen/cell type	Isotype	Source ^a	Use ^b	Reference
MM1A	CD3	IgG1	VMRD, Inc., Pullman, WA	MACS	18
CC30	CD4	IgG1	IAH	FACS, IF	9
CC8	CD4	IgG2a	IAH	MACS, FACS, IF	41
CC21	CD21	IgG1	IAH	MACS, FACS	53
CCG33	CD14	IgG1	IAH	MACS, FACS	61
CC149	CD172 α	IgG2b	IAH	MACS, FACS	38
CC108	MHC II	IgG1	IAH	FACS	39
CC76	CD45RB	IgG1	IAH	FACS, IF	42
TRT1	Turkey (rhinotracheitis virus)	IgG1		FACS, IF	17
TRT3	Turkey (rhinotracheitis virus)	IgG2a		FACS, IF	17
ILA-16	CD11c	IgG1	ILRAD	MACS, FACS	40
BF2	IFN- α	IgM		IF	11
GB21A	δ -Chain receptor	IgG2b	VMRD, Inc., Pullman, WA	MACS	13
Gr13.1	Anti-ovine NKp46; NK cells	IgG1	Timothy Connelly, University of Edinburgh	MACS, FACS	Unpublished
2C2	FMDV 3A	IgG2a		IF	12
AV48	Chicken antigen	IgM	IAH	IF	17

^a IAH, Institute for Animal Health; ILRAD, International Laboratory for Research on Animal Diseases.

^b MCS, magnetic cell sorting; FACS, fluorescence-activated cell sorting; IF, immunofluorescence.

starting from a model including tissue, cell source/antigen, and an interaction between these factors.

Analysis of the significance of the levels of type I IFN produced *in vivo* by CD4-depleted and non-CD4-depleted animals was carried out as follows. Changes in type I IFN levels (y) over time (t) were described using the following function:

$$y(t) = \delta + \alpha \left(\frac{t}{\beta} \right)^{\beta^2/\gamma^2} \exp \left(- \frac{\beta(t - \beta)}{\gamma^2} \right)$$

where δ is the baseline IFN level, α is the maximum increase in IFN above baseline, β is the time at which the maximum IFN level occurs, and γ is the time before (after) the maximum IFN level at which the maximum rate of increase (decrease) in the IFN level occurs. Parameters for each animal were estimated by fitting the curve described above to the data using the method of least squares. Estimates for each animal were then compared using Kruskal-Wallis tests to identify significant ($P < 0.05$) differences among CD4-depleted and non-CD4-depleted animals.

RESULTS

Comparison of type I IFN levels produced by large low-density cells from blood, pseudoafferent lymph, and mesenteric lymph nodes. pDCs are large low-density (LLD) cells found in various tissues in low numbers, including blood, lymph nodes (23), afferent lymph (58), lungs (19), liver (1), and skin (67). To date, no such information has been published for bovine pDCs. To identify a bovine tissue from which we could isolate pDCs, LLD cells were first prepared from blood, pseudoafferent lymph, and mesenteric lymph nodes by using density gradient centrifugation. Next, we used a Mx-CAT reporter assay to determine the respective capacities of the LLD cells to produce biologically active type I IFN before and after incubation with FMDV or FMDV-immune complexes (32). Unmethylated CpG oligodeoxynucleotides were used as a positive control for the stimulation of type I IFN (59). In agreement with published data, FMDV alone stimulated little, if any, type I IFN in LLD cell populations from all three tissues (35). In comparison to incubations with FMDV alone, FMDV-immune complexes induced large quantities of type I IFN in cells from all three tissues. Interestingly, LLD cells isolated from mesenteric lymph nodes produced significantly larger (P , 0.005 and

0.017, respectively) amounts of type I IFN than LLD cells from blood and pseudoafferent lymph and were therefore used in further experiments to purify and characterize bovine pDCs (Fig. 1A, B, and C).

Enrichment of type I IFN-producing cells following selective depletion. To enrich the population of pDCs present in the LLD cells from mesenteric lymph nodes, we performed magnetic cell sorting using a panel of antibodies to deplete other cell types. Initially, appropriate monoclonal antibodies (Table 1) were used to deplete CD3⁺ T cells, CD21⁺ B cells, and CD14⁺ monocytes/macrophages. Each antibody used to perform the depletion has been characterized previously (see Materials and Methods), and similar approaches have facilitated the enrichment of pDCs from porcine blood and sheep lymph (35, 58, 63). The purity of the enriched population of type I IFN-producing cells was determined by FACSCalibur analysis, confirming the depletion (>97% in all cases) of CD3⁺, CD21⁺, and CD14⁺ cell populations (Fig. 2A). Mx-CAT reporter assays showed that the enriched populations of cells produced significantly more type I IFN than the nonenriched populations (P , <0.001 for total MLN compared to LLD cells and 0.0014 for LLD cells compared to depleted populations) in response to FMDV-immune complexes (Fig. 2B).

Further experiments were performed, revealing that the pDC populations responsible for the observed production of type I IFN were NKp46⁺ and TCR δ ⁺ cells (natural killer [NK] cells and gamma delta [$\gamma\delta$] T-cell receptors [TCR], respectively) (Fig. 3A).

Depletion of CD4 *in vitro*. Both human and porcine pDCs have been phenotypically characterized by CD4 receptor expression (35, 64). Enrichment for CD4⁺ cells was attempted (data not shown), but receptor cross-linking using MACS technology led to inhibition of type I IFN production. This phenomenon has been described previously by Fanning et al., who showed that cross-linking of CD4 inhibited IRF-7 translocation to the nucleus (28). However, depletion of CD4⁺ cells from the enriched pDC population almost completely abolished type I IFN production (Fig. 3A). Taken together, these

FIG. 1. Type I IFN generated by large low-density (LLD) cells from three different tissues in response to FMDV-immune complexes (IC) and CpG. Shown are results for LLD cells isolated from bovine blood (A), bovine pseudoafferent lymph (B), and bovine mesenteric lymph nodes (C). The results from three individual experiments are shown for each tissue.

results characterize bovine lymph node pDCs as CD3[−] CD4⁺ CD14[−] CD21[−] TcR1-N24[−] (TCR δ chain) NKp46[−] (NK) cells.

pDCs express toll-like receptors (TLR7 and -9) and constitutively express the transcription factor IRF-7, which facilitates a rapid and robust IFN response to viral infection (31). We therefore incubated LLD cells, isolated from bovine lymph nodes and depleted of cells expressing CD3, CD21, CD14, NK p46, and $\gamma\delta$ T-cell receptor, with FMDV, FMDV-immune complexes, or CpG and analyzed whole-cell lysates for IRF-7 protein. In agreement with the IFN assays, incubation of FMDV-immune complexes or CpG led to an increase in the level of IRF-7 protein; however, no such increase was observed with FMDV alone (Fig. 3B).

Depletion of CD4⁺ cells *in vivo*. The anti-CD4 monoclonal antibody (CC8) used to selectively deplete CD4⁺ LLD cells from the enriched pDC population (see the data presented above) was successfully used by Juleff and coworkers, along with monoclonal antibody IL-A11, to deplete circulating CD4⁺ cells in calves prior to infection with FMDV (43). However, the previous study focused on the role of CD4⁺ T-cell-independent antibody responses in the resolution of FMD. In order to gain insight into the effect of CD4⁺ cell depletion on the production of type I IFN during the early stages of FMDV infection (0, 1, 2, 3, 4, 5, 6, and 7 days postinfection [dpi]), the serum samples taken during this study were reanalyzed. For each control calf, a peak of type I IFN production was observed between 1 and 4 dpi. In comparison to all six calves not depleted of CD4⁺ cells, three of the four CD4-depleted calves exhibited significantly lower quantities of type I IFN in serum. There were significant differences between CD4-depleted and non-CD4-depleted cattle in terms of the maximum increase in the level IFN above baseline ($P = 0.04$), but not in terms of the

baseline IFN level ($P = 0.21$), the time at which maximum IFN production occurred ($P = 0.43$), or the time at which the maximum rate of increase occurred ($P = 0.55$) (Fig. 3C). In agreement with the results of the *in vitro* depletion studies presented above, these results show a positive correlation between the presence of bovine CD4⁺ cells and the production of type I IFN.

Comparison of the levels of type I IFN produced by CD11c[−] and CD11c⁺ cells. Due to the ambiguity of CD11c expression in other species, we decided to investigate its expression on the surfaces of bovine pDCs. As previously described, LLD cells isolated from bovine lymph nodes were first depleted of CD3⁺, CD14⁺, and CD21⁺ cell populations. Further magnetic cell sorting was performed to separate the constituent CD11c⁺ and CD11c[−] cell populations (Fig. 4A). The CD11c⁺ and CD11c[−] cell populations were then incubated with FMDV, FMDV-immune complexes, or CpG, and the resulting supernatants were assayed for type I IFN (Fig. 4B). In comparison to the CD11c⁺ cells, the CD11c[−] cells produced significantly more type I IFN in response to both FMDV-immune complexes and CpG ($P, 0.0077$ and 0.0123 , respectively). These results clearly indicated that the enriched population of bovine pDCs is CD11c[−].

Bovine plasmacytoid dendritic cell FACS phenotype. The selective depletions used to enrich for the population of bovine pDCs confirmed the cells to be CD3[−], CD14[−], CD21[−], NKp46[−], TCR δ [−], CD11c[−], and CD4⁺. To identify other surface molecules expressed on these pDCs, FACS analysis was performed. Total (nondepleted) LLD cells were triple stained for the combined expression of CD3, CD4, and either major histocompatibility complex class II (MHC II), CD45RB, CD172 α , or CD32. Analysis of the CD3[−] and CD4⁺-gated

FIG. 2. Sequential enrichment of type I IFN-producing cells by density gradient centrifugation and lineage-specific cell population depletion. (A) FACS analysis of cell populations before (top) and after density gradient centrifugation to enrich for LLD cells (center), followed by depletion of CD3⁺, CD14⁺, and CD21⁺ cells using Miltenyi MACS technology (bottom). (B) Biologically active type I IFN produced by LLD cells at each step of the enrichment after stimulation with FMDV, FMDV-immune complexes (IC), or CpG. Mx-CAT reporter assays showed that enriched populations of cells produced significantly more type I IFN than nonenriched populations (*P*, <0.001 for total MLN compared to LLD cells; *P*, 0.0014 for LLD compared to depleted cells) in response to FMDV-immune complexes. The results from three individual experiments are shown for each tissue.

population of total LLD cells confirmed the pDC to be MHC II⁺, CD45RB⁺, CD172α⁺, and CD32⁺ (Fig. 5A).

In addition to the data presented above, confocal microscopy carried out on the depleted population (CD3[−] CD14[−]

CD21[−] NKp46[−] TCR δ chain[−] CD11c[−]) after incubation with CpG revealed that the bovine pDCs exhibited dendritic cell morphology, were CD45RB⁺ and CD4⁺, and expressed IFN-α protein (Fig. 5b). In pDCs stimulated with CpG and treated

FIG. 3. Characterization of the cells producing type I interferon *in vitro* and *in vivo*. (A) Effects of *in vitro* depletion of NK cells, $\gamma\delta$ T cells, and CD4⁺ cells on the production of type I IFN from cells prepared from mesenteric lymph nodes. Depletion of CD3⁺, CD14⁺, CD21⁺, NK, and $\gamma\delta$ T cells significantly enriched type I IFN production in response to FMDV-immune complexes (IC) or CpG [Depleted (+CD4)]. However, depletion of CD4⁺ cells almost completely abolished type I IFN production in response to the same stimuli [Depleted (-CD4)]. (B) Western blot analysis of the level of IRF-7 after stimulation of a cell population depleted of CD3⁺, CD14⁺, CD21⁺, NK, and $\gamma\delta$ T cells with immune serum (Ab only), FMDV, FMDV-immune complexes (IC), or CpG. Levels of IRF-7 increased in response to FMDV-immune complexes or CpG but not in response to virus alone, immune serum, or no treatment. Note that IRF-7 could not be detected in LLD cells prior to depletion enrichment. (C) Levels of type I IFN in serum from FMDV-infected cattle depleted of CD4⁺ cells compared to non-CD4⁺-depleted controls (including $\gamma\delta$ and CD8-depleted animals). The *in vitro* depletion studies presented show a positive correlation between the presence of bovine CD4⁺ cells and the production of type I IFN in response to FMDV-immune complexes, comparing large low-density (LLD) cells and CD3⁺, CD14⁺, CD21⁺, NK, and $\gamma\delta$ -depleted populations to the CD4⁺-depleted population (*P*, 0.0019 and 0.013, respectively).

FIG. 4. Comparison of the level of type I IFN produced by CD11c⁻ and CD11c⁺ cells. (A) FACS analysis of LLD cells depleted of CD3⁺, CD14⁺, and CD21⁺ cells and then separated into CD11c⁻ and CD11c⁺ cell populations. (B) The levels of biologically active type I IFN produced by the CD3⁺, CD14⁺, and CD21⁺ cell-depleted population of LLD cells (Depleted) in response to immune serum (serum), FMDV (virus), FMDV-immune complexes (IC), or CpG were compared to those for the separated CD11c⁻ [Depleted (CD11c⁻)] and CD11c⁺ [Depleted (CD11c⁺)] populations using the Mx-CAT reporter assay. CD11c⁻ cells produced significantly more type I IFN in response to FMDV-immune complexes or CpG (*P*, 0.0077 and 0.0123, respectively). The results from three individual experiments are shown for each tissue.

FIG. 5. Phenotype of CD4⁺ CD3⁻ cells capable of expressing type I IFN in response to CpG. (A) FACS analysis of a CD4⁺ CD3⁻ cell population showing the expression of CD172a, MHC II, CD11c, CD45RB, and CD32 by staining. (B) Immunostaining and confocal microscopy carried out on CpG-stimulated cells. (a) pDCs were stimulated for 4 h with CpG in the presence of brefeldin A to trap type I IFN in the ER and were then immunostained for type I IFN. (b) pDCs were stimulated for 16 h with CpG and were then immunostained for type I IFN and CD4. (c) pDCs stimulated for 16 h with CpG were immunostained for CD45RB and CD4 surface expression. (d) pDCs stimulated for 16 h with CpG were immunostained with IgM as an isotype control. (e) pDCs stimulated for 16 h with CpG were immunostained with IgG1 as an isotype control.

with brefeldin A, the IFN- α was retained in the endoplasmic reticulum (ER) (Fig. 5B).

Live FMDV is necessary for the induction of type I IFN in bovine pDCs. Guzylack-Pirou et al. have shown that in the pig, type I IFN production by pDCs in response to FMDV requires immune complexes and live virus (35). Having determined that FMDV-immune complexes are also required for the production of type I IFN by bovine pDCs, we decided to investigate the requirement for live virus. For this purpose, immune complexes, made using live FMDV O₁K, UV-inactivated O₁K, or empty capsids (O₁ Manissa), were incubated with enriched pDC populations from two animals, and the levels of type I IFN generated were determined using the Mx-CAT reporter assay. Repeated experiments clearly showed a requirement for live virus in the induction of type I IFN, since immune com-

plexes formed with UV-inactivated virus or empty capsids were unable to elicit type I IFN production (Fig. 6A). Furthermore, after incubation with live-virus-immune complexes, CD4⁺ non-T cells exhibiting expression of the nonstructural protein 3A were observed, suggesting that replication was initiated within these cells (Fig. 6B).

DISCUSSION

In this study we have characterized a bovine cell population that is lineage negative and CD4⁺ and corresponds to the pDC populations described for other species. pDCs are described as large low-density cells, and our initial experiments comparing tissue prevalence indicated that a higher number are present in secondary lymphoid tissue. The sequential enrichment of

FIG. 6. Type I IFN produced in response to FMDV-immune complexes requires replication-competent/replicating virus. (A) Type I IFN produced by pDC after incubation with either CpG, FMDV (virus), FMDV-immune complexes of live virus (IC), UV-inactivated virus (I-virus), empty capsids (EC), or empty capsids in immune complexes (EC-IC). The results from two individual experiments are shown. (B) Immunostaining of pDCs for FMDV nonstructural protein 3A and CD4. (a) Infected pDCs stained positive for both CD4 and 3A. (Top) DIC image overlays; (bottom) CD4 immunofluorescence (green) and 3A staining (red). (b) Uninfected pDCs stained positive for CD4 but negative for 3A. (Top) DIC image overlay; (bottom) CD4 immunofluorescence (green) and no positive staining for 3A (red).

pDCs by density gradient centrifugation and selective depletion of specific populations of cells partially defined the phenotype of bovine pDCs as $CD3^- CD14^- CD21^- NKp46^- TCR\ \delta\ chain^-$ (expressed on $\gamma\delta$ T cells). Immunostaining and FACSCalibur analysis further defined the population as $CD4^+ CD45RB^+ MHC\ II^+ CD172a^+ CD32^+ CD11c^-$. Confocal microscopy on immunostained pDCs following treatment with CpG was carried out to confirm the presence of type I IFN in treated cells and the surface phenotype of those cells. Following activation with CpG, pDCs displayed a dendritic phenotype but retained CD4 and CD45RB expression on the cell surface. This is in agreement with the data published by Pascale et al. (58), describing the phenotype of ovine pDCs. In our hands, experimental depletion of $CD4^+$ cells *in vitro* significantly re-

duced the level of type I IFN produced. Likewise, in three of four cattle depleted of $CD4^+$ cells, we observed a significant reduction in the serum type I IFN level during the early stages of FMDV infection. Interestingly, the depletion of $CD4^+$ cells, which resulted in a profound reduction in the circulating type I IFN level, had no adverse effect on the magnitude or duration of clinical signs, the induction of neutralizing antibodies, or the clearance of virus from the circulation (43). The pattern of type I IFN expression *in vivo* is consistent with *in vitro* observations. Even though the virus is present in blood, type I IFN is not induced until, presumably, T-independent antibodies are produced and immune complexes form. The timing of type I IFN induction after the development of neutralizing antibodies also suggests that this cytokine does not play a major role in con-

trolling infection. In contrast, exogenous IFN has a potent effect when administered prior to infection (66).

In all species where pDCs have been characterized, it is now generally accepted that these cells are CD4⁺ non-T cells and that their depletion would be expected to significantly reduce the type I IFN response to viral infection and known TLR7/9 agonists. To date, *in vivo* studies examining the effects of pDC depletion on type I IFN production have been mainly restricted to the murine model and have depleted pDCs using cell-specific monoclonal antibodies other than anti-CD4. Asselin-Paturel et al. demonstrated that *in vivo* depletion of pDC in mice, using a monoclonal antibody specific for murine pDC, led to a significantly reduced capacity to produce IFN- α in response to CpG (5). Similarly, Barchet et al. have shown, using a MyD88^{-/-} mouse model, that the IFN- α response to TLR7 agonists *in vivo* is completely absent (7). Using the anti-mPDCA-1 monoclonal antibody to deplete pDCs *in vivo*, they showed that the levels of serum IFN- α produced in response to the known TLR7 agonists GU-rich oligoribonucleotides (ORN) are significantly reduced and that pDCs are entirely responsible for the type I IFN produced. So far, two TLRs have been identified in pDC activation in response to viral infection: TLR7 and TLR9. TLR7 is a receptor for single-stranded RNA (ssRNA) and is involved in the recognition of RNA viruses such as influenza virus (24), whereas TLR9 recognizes CpG motifs found in the genomes of DNA viruses, such as herpesviruses (36). In a more recent study, Zhang et al. (68) have cloned and expressed porcine TLR7 cDNA in HEK293 cells and have shown that immunostimulatory RNA ORN found in FMDV activated NF- κ B and induced the expression of IFN- α via TLR7. Taken together, these data suggest a role for the endosomal RNA receptor TLR7 in the initiation of type I IFN production in pDCs in response to FMDV. This hypothesis is supported by the findings of Guzylack-Pirou et al., which describe the inhibition of IFN- α production in porcine pDCs by the administration of chloroquine, which neutralizes the acidic environment of endocytic vesicles, but not by leupeptin, ruling out the involvement of lysosomes (35). Their results also suggest the involvement of an RNA receptor associated with endocytic compartments, such as TLR7, although further studies are required to clarify this mechanism.

The importance of pDCs and their ability to rapidly secrete large quantities of IFN- α in response to many viral infections has been well documented. pDCs can induce high levels of type I IFN in response to many viruses irrespective of the state of infectiousness. The coronavirus transmissible gastroenteritis virus (TGEV) is an example of live or inactivated virus stimulating equally well (15). Herpes simplex virus is yet another example where the state of infectiousness is irrelevant to the induction of type I IFN (3). Both these viruses are enveloped, and the induction of type I IFN is related to their glycosylated viral envelope proteins. In contrast, *in vitro* studies indicate that pDCs have a limited capacity to respond to many nonenveloped viruses directly, or fail to do so (30). To date, there is no demonstrated explanation of the differing responses to enveloped and nonenveloped viruses, though the differing mechanisms of cell entry could possibly result in different subcellular localization and processing. Porcine pDCs have been demonstrated to produce little if any type I IFN in response to FMDV *in vitro* (35). However, this study also defines the requirement

for FMDV-immune complexes in the activation of pDCs and reports the requirement for live virus for this activation to occur. We also found that virus-immune complexes of live FMDV were necessary to activate bovine pDCs; immune complexes of UV-inactivated virus or empty viral capsids were barely able to elicit type I IFN production. The lack of type I IFN production by FMDV alone suggests that these cells do not express the appropriate receptors to permit infection; however, Fc receptor-mediated uptake of immune complexes permits the virus access to cells that would otherwise be inaccessible.

Human pDCs have been reported to express CD32 (Fc γ R11a) (8, 49). Means et al. showed that DNA-containing immune complexes (ICs) within lupus serum (SLE-ICs), but not protein-containing ICs from other autoimmune rheumatic diseases, could stimulate pDCs to produce cytokines and chemokines via a cooperative interaction between TLR9 and Fc γ RIIa (CD32) (49). Similarly, human pDCs exhibit weak expression of CD32, but not other Fc receptors, such as CD16 and CD64 (47). Porcine pDCs have also been reported to express CD32. Balmelli et al. describe the colocalization of CD32 with classical swine fever virus (CSFV) in polarized structures in "immune pDC" and attribute immune complex formation to cytophilic antibodies bound to Fc γ RIIa (6). Guzylack-Pirou et al. reported the ligation of CD32 by FMDV-immune complexes in porcine pDCs (35). We also detected Fc γ RIIa expressed on CD4⁺ CD3⁻ cell populations from bovine mesenteric lymph nodes and have shown that antibody-mediated uptake of replication-competent virus in immune complexes is required to initiate the production of type I IFN in these cells.

The differential expression of CD11c has the potential to differentiate myeloid dendritic cells (mDCs) from pDCs in mixed cell populations. CD11c, also known as the integrin alpha X chain protein, when combined with the beta 2 chain (ITGB2), forms a leukocyte-specific integrin referred to as inactivated C3b (iC3b) receptor 4 (CR4). This protein is commonly regarded as a myeloid lineage marker, expressed highly on most dendritic cells, monocytes, macrophages, and neutrophils. However, the expression of this protein on pDCs of different species is variable. Murine pDCs express CD11c (4, 10, 54), whereas human pDCs are negative for this myeloid marker (33, 62), as are ovine pDCs (58). Porcine pDCs have been reported as positive for CD11c (63); however, a recent publication by Calzada-Nova et al. determined this molecule to be absent from porcine pDCs (14). Here, in agreement with the majority of the data published for other species, we show that bovine pDCs do not express CD11c.

In summary, we have defined the phenotype of bovine pDCs to be CD3⁻ CD14⁻ CD21⁻ NKp46⁻ TCR δ ⁻ CD11c⁻ CD4⁺ CD45RB⁺ MHC II⁺ CD172a⁺ CD32⁺. We believe this to be the first description of a bovine cell phenotype that corresponds to and behaves as pDCs described for other species. We have also shown that initiation of type I IFN production by these cells in response to FMDV requires immune complex formation and at least the initial stages of viral replication.

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