The Double-Stranded RNA Bluetongue Virus Induces Type I Interferon in Plasmacytoid Dendritic Cells via a MYD88-Dependent TLR7/8-Independent Signaling Pathway

Suzana Ruscanu,* Florentina Pascale,†,‡ Mickael Bourge,§ Behzad Hemati,a* Jamila Elhouzzi-Younes,a Céline Urien,a Michel Bonneau,b Haru Takamatsu,a Jayne Hope,a Peter Mertens,d Gilles Meyer,a Meredith Stewart,a Polly Roy,a Eliane F. Meurs,h Stéphanie Dabo,h Stéphan Zientara,a Emmanuel Beaud,a Corinne Saillieu,a Emilie Chauveau,a Damien Viotur,a Bernard Charley,a and Isabelle Schwartz-Cornil*a

Virologie et Immunologie Moléculaires, UR892 INRA, Jouy-en-Josas, France; Centre de Recherche en Imagerie Interventionnelle, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; IFR87 La Plante et son Environnement; IMAGIF CNRS, Gif sur Yvette, France; Vector Bourne Viral Disease Programme; Institute for Animal Health, Woking, Surrey, United Kingdom; Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, United Kingdom; Université de Toulouse, INP, ENVT, INRA UMR1225, IAHP, Toulouse, France; London School of Hygiene and Tropical Medicine, London, United Kingdom; Institut Pasteur, Hepacivirus and Innate Immunity, Paris, France; and UMR 1161 ANSES/INRA/ENWA, Maisons-Alfort, France

Dendritic cells (DCs), especially plasmacytoid DCs (pDCs), produce large amounts of alpha/beta interferon (IFN-α/β) upon infection with DNA or RNA viruses, which has impacts on the physiopathology of the viral infections and on the quality of the adaptive immunity. However, little is known about the IFN-α/β production by DCs during infections by double-stranded RNA (dsRNA) viruses. We present here novel information about the production of IFN-α/β induced by bluetongue virus (BTV), a vector-borne dsRNA Orbivirus of ruminants, in sheep primary DCs. We found that BTV induced IFN-α/β in skin lymph and in blood in vivo. Although BTV replicated in a substantial fraction of the conventional DCs (cDCs) and pDCs in vitro, only pDCs responded to BTV by producing a significant amount of IFN-α/β. BTV replication in pDCs was not mandatory for IFN-α/β production since it was still induced by UV-inactivated BTV (UV-BTV). Other inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-12p40, were also induced by UV-BTV in primary pDCs. The induction of IFN-α/β required endo-/lysosomal acidification and maturation. However, despite being an RNA virus, UV-BTV did not signal through Toll-like receptor 7 (TLR7) for IFN-α/β induction. In contrast, pathways involving the MyD88 adaptor and kinases dsRNA-activated protein kinase (PKR) and stress-activated protein kinase (SAPK)/Jun N-terminal protein kinase (JNK) were implicated. This work highlights the importance of pDCs for the production of innate immunity cytokines induced by a dsRNA virus, and it shows that a dsRNA virus can induce IFN-α/β in pDCs via a novel TLR-independent and Myd88-dependent pathway. These findings have implications for the design of efficient vaccines against dsRNA viruses.
viruses in vivo, the use of hematopoietic mouse chimeras revealed that hematopoietic cells, including both cDCs and plasmacytoid DCs (pDCs), play a key role in type I IFN production during orbivirus infection (34). Indeed, pDCs are the main hematopoietic cell type specialized in the production of innate cytokines and, especially, of huge amounts of IFN-α/β. PDC responses to viral triggers were studied in single-stranded RNA (ssRNA) and DNA virus infections. TLR7 and TLR9 linked to the MyD88 adaptor molecule were demonstrated to be the major and possibly the only innate receptors that activate this cell type. However, cytoplasmic DExD/H-box helicases linked to MyD88 can also be involved in viral DNA recognition in pDCs (36). Both orbivirus infection in mice and rotavirus infection in human are capable of triggering the production of IFN-α/β in pDCs. However, as pDCs do not express TLR3 (14, 48) and do not use the RIG-I/MDA5 signaling pathway (35, 54, 63), it is still not known how pDCs respond to dsRNA virus infection. In addition, very few studies have been reported to date concerning the relative contribution of pDCs to innate cytokine production in response to dsRNA virus infection.

In this study, we addressed the role of pDCs in IFN-α/β production in response to BTV compared to the role of other hematopoietic cells, including the cDCs that represent the initial cell target for BTV replication after its injection into sheep skin. In sheep, pDCs are rare cells found in blood (<0.5%), and they also circulate in skin lymph (52). Preliminary experiments in C57BL/6 mice indicated that murine hematopoietic cells and purified murine pDCs do not respond to BTV by producing IFN-α/β, preventing the use of mouse models to address BTV signaling for IFN-α/β production. We therefore directly investigated the interaction of BTV with its natural sheep host pDCs and cDCs, and we were able to use primary cells, due to the large quantities of cells that can be collected in this species.

MATERIALS AND METHODS

Medium and reagents. X-Vivo 15 (BioWhittaker) supplemented with 1% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin was used as the cell culture medium for sheep cells. RPMI 1640 supplemented with 4% horse serum (HS) was used for cell immunolabeling (fluorescence-activated cell sorting [FACS] medium). Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin was used for baby hamster kidney-21 (BHK21) culture (virus growth and titration). Eagle’s minimum essential medium (EMEM) supplemented with 5% FCS, 1.5 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin was used for Madin-Darby bovine kidney (MDBK) cell culture. CpG-A D32-oligodeoxynucleotide with the sequence ggt CCG TCG ACG CAG ggg gg (lowercase letters for phosphohioate linkages and uppercase letters for phosphodiester linkages) was produced by BioSource International. Opti-MEM (Invitrogen) and Lipofectamine 2000 (Invitrogen) were used for poly(1-C) (Sigma-Aldrich) transfection, 2-Aminopurine (2-AP), chloroquine, and bafilomycin A1 were obtained from Sigma-Aldrich. SP600125 (Jun N-terminal protein kinase [JNK] inhibitor) and PKR inhibitor (C16) were from Calbiochem (Merck), and PD184352 was from AXON Medchem. The MyD88 homodimerization inhibitory peptide set was purchased from Imgenex. The A151 oligonucleotide (TTAGGG), a TLR7/9 antagonist (26), was synthesized at the Pasteur Institute Genopole, Paris, France.

Virus preparations. Wild-type field strains of BTV serotypes 1, 2, and 8 (BTV1, BTV2, and BTV8) were isolated in France in 2007, 2001 (Corsica), and 2006, respectively. These viruses are still virulent and pathogenic in sheep. The modified live attenuated vaccine strain BTV2 (a-BTV2) was produced by Ondersteoport Biological Products in the Republic of South Africa. For purified BTV1, virus particles of the South African reference strain of BTV1 were purified on sucrose gradients as previously described by Mertens et al. (46). This strain of BTV is held in the Institute for Animal Health (IAH, United Kingdom) reference collection (www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-1.htm) and is identified by strain number RSArrrr/01.

Viruses were grown on BHK21 cells. For preparation of crude viral stocks, confluent BHK21 cell layers were infected with 0.1–0.5% tissue culture infective dose (TCID50)/cell and cells were incubated at 37°C and 5% CO₂. When cytopathic effect was complete (4 to 5 days), culture supernatants were aliquoted and stored at ~80°C. The TCID50 values were estimated by the method of Spearman-Karber using a previously described protocol (27).

Heat inactivation of BTV was done at 56°C for 30 min. For UV inactivation, virus preparations (2 ml) were exposed to 254-nm UV at a 10-cm distance from the source in 35-mm-diameter dishes. Virus was completely inactivated (UV–BTV) in 20 min when the dose of UV was 2.3 J/cm² (see Fig. S2 in the supplemental material). Formol-inactivated influenza virus (PR8/34) was prepared as previously described (61).

Animals and afferent lymph and blood collection. Preadip BTV-seronegative female sheep, originating from the Unité Commune d’Expérimentation Animale in Jouy-en-Josas, France, were catted at the Centre de Recherche en Imagerie Interventionnelle in Jouy-en-Josas as previously described (23). Low-molecular-weight heparin (enoxaparin [Lovenox], Sanofi-Aventis) was injected intradermally into the shoulder skin every 12 h (2,000 IU anti-enzaparin per injection). Lymph was collected twice a day in flasks containing 500 IU heparin, 10,000 IU penicillin, and 10 mg streptomycin. This protocol was carried out in strict accordance with the recommendations of the Charte Nationale Portant sur l’Éthique en Expérimentation Animale established by the Comité National de Réflexion Éthique sur l’Expérimentation Animale (CNREEA, Ministère de l’Enseignement Supérieur et de la Recherche et Ministère de l’Agriculture et de la Pêche) and was approved by the Committee on the Ethics of Animal Experiments of the INRA research center in Jouy-en-Josas and Agroparistech under the number 11-019. The animal experiments were carried out under licenses issued by the Veterinary Services of Versailles (accreditation numbers A78-93, A78-15, and A78-730).

Blood was collected from Prealp sheep (1 to 4 years old) by venous puncture on sodium citrate.

In vivo infections. Two cannulated sheep were inoculated intradermally with 10⁶ TCID₅₀ a-BTV2 and BTV8, respectively, in the biosafety level 3 (BSL3) animal facilities of the Centre de Recherche en Biologie Médicale in Maisons-Alfort, France. Lymph draining the corresponding area of the skin was harvested at different time points as indicated below, and the sheep were terminated at the end of the experiment.

For IFN-α/β detection in sera, BTV-seronegative Prealp sheep were infected subcutaneously and intravenously with, respectively, 1 and 5 ml of blood from a viremic BTV8-infected animal (8.1 × 10⁸ BTV RNA copies/ml) in the BSL3 facilities of the Plate-Forme d’Infectiologie Expérimentale in Nouzilly, France. Sera were obtained from the infected animals at 0, 2, 6, and 10 days after infection. The sheep were terminated at the end of the experiment.

Low density (LD) lymph and LD peripheral blood mononuclear cell (PBMC) isolations. Total afferent lymph cells were spun down at 700 × g, and LD lymph cells were obtained after centrifugation on a 1.065-g/ml iodixanol density gradient (OptiPrep, Nycomed Pharma) as previously described (57).

PBMCs were obtained from sheep peripheral blood buffy coat samples by 1.076-g/ml Percoll (GE Healthcare) density gradient centrifugation (8). LD PBMCs were obtained after centrifugation on iodixanol gradient as described for lymph cells.

In vitro activation of LD lymph cells, LD PBMCs, cDCs, and pDCs with BTV and IFN-α/β inducers and use of inhibitors. LD lymph cells, LD PBMCs, cDCs, and pDCs were incubated at 37°C with 0.01 to 0.5 TCID₅₀ BTV/cell in cell culture medium. The cell and viral concentrations used in...
overnight with BTV. For detection of intracellular NS2 and surface antibodies were consistently used as controls to measure the level of nonspecific background signal caused by primary antibodies.

Cell viability was determined by staining nonviable cells with 7-amino actinomycin D (7-AAD; 2 μg/ml), and flow cytometry analysis was performed to measure the percentage of surviving (7-AAD-negative) cells.

The pDCs and cDCs (between 2 × 10^5 and 5 × 10^5 per 500 ml of blood collected) were sorted by flow cytometry on the LMaGif cytometry platform using the analyzer-sorter MoFlo XDP cytometer and the Summit 5.2 software from Beckman Coulter (purity of >98%). Due to the low number of purified pDC that could be obtained, only single experimental points could be done per sheep. The validity of the results was demonstrated by repeating these single experimental points using different sheep (see figure legends). The flow cytometry analysis of NS2 and CD80/86 expression in pDCs was done using a FACS Calibur cytometer (Becton Dickinson) with the CellQuest Pro and/or FlowJo software.

IFN-α/β detection (bioassay) and ruminant IFN-α ELISA. IFN-α/β in cell supernatants, lymph fluids or sera was quantified using a cytopathic effect reduction bioassay with MDBK cells challenged with vesicular stomatitis virus. An internal IFN-α reference was included as described elsewhere (52). Each supernatant was tested over eight serial dilutions. Data are expressed as IFN-α/β units per ml. A specific bovine/ovine IFN-α enzyme-linked immunosorbent assay (ELISA) was used as described previously using anti-bovine IFN-α 1C6 and 1D10 MAbS and rabbit anti-bovine IFN-α IgG (52).

Cytokine mRNA detection by qRT-PCR. Total RNA was extracted using the Arcturus PicoPure RNA isolation kit (Arcturus). To exclude DNA contamination, RNA samples were treated with RNase-free DNase (Qiagen). cDNA was prepared using SuperScript III RT (Invitrogen). Quantitative real-time PCR was done using 5 ng cDNA with 300 nM primers in a final reaction mixture volume of 25 μl 1 × SYBR green PCR master mix (Applied Biosystems) using an Eppendorf Mastercycler ep realplex system.

The primers used were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, CACCATCTTCCAGGAGCGAG, and reverse, CCA GCACTACCCCACTGTGA), IFN-α (forward, TCTGCAAGAAGG ACACA, and reverse, GCTGCAAGTGTGAGAGG), tumor necrosis factor (TNF) (forward, CAAGGGCCAGGGTTCTTACC, and reverse, GCCCAACCATGCTAAGTCTT), IL-6 (forward, GCTGCTCTCTGGTA TGACTCT, and reverse, GGTGGTGTCACTTTTGAACATCTT), and IL-12 (forward, TCTCGGACAGTGGAAGTCA, and reverse, ACTTTG GTGAGGTTGTGTC). Quantitative analysis was performed using the Realplex software (Eppendorf). Cytokine mRNA levels relative to the amount of GAPDH were calculated by analyzing the cycle threshold (Ct) values for each amplification curve, and the arbitrary units were established by the formula 2^-ΔΔCt.

Viral RNA detection was done using a commercial pan-BTV RT-PCR (Adiavet BTV real-time A352; Adiagene, France) targeting segment 10 of BTV, which is highly conserved among BTV serotypes.

Statistical analyses. Due to the low numbers of purified pDCs that could be collected from 500 ml of blood in many instances, replicates and statistics could not be done within an experiment, but the experiments were repeated with different animals. When replicates were possible, data were expressed as means ± standard errors of the means (SEM). Statistical significance of differences was determined by paired or unpaired Student’s t tests, by a paired one-tail Wilcoxon test, or by a Kruskal-Wallis test. The test used is mentioned in the figure legends (in the figures, one asterisk shows a P value of <0.05 and two asterisks show a P value of <0.005). Differences were considered statistically significant for P values of <0.05. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS
Administration of BTV in sheep induces IFN-α/β in afferent skin lymph and in blood. We first examined whether BTV induces IFN-α/β in vivo in sheep. We took advantage of the possibility to sequentially collect afferent skin lymph in this species after virus inoculation in the skin (10, 27). Inoculation of attenuated BTV2 (a-BTV2) and wild-type BTV8 (BTV8) in the skin in-
duced IFN-α/β production in lymph, peaking at day 6 with a-BTV2 (16 IU/ml) and at day 5 with BTV8 (12 IU/ml), which corresponds to the peak of viral dissemination by cDCs from skin (27) (Fig. 1A and B). IFN-α/β (Fig. 1A and B) and BTV (data not shown) were undetectable in lymph by day 10. We also found circulating IFN-α/β in the blood of sheep inoculated with BTV8 at days 2 and 6 postinjection (Fig. 1C). IFN-α/β reached basal levels in blood at day 10 postinjection. The viral load in blood peaked at days 2 and 6 postinjection (Fig. 1C). IFN-α/β was measured in the lymph fluid (U/ml) using a bioassay. (C) Sheep (n = 5) were infected intravenously and subcutaneously with 1 and 5 ml blood, respectively, from a BTV8-infected viremic sheep. IFN-α/β in sera was measured using a bioassay. Significant differences between groups are indicated (*) P < 0.05; unpaired t test).

**FIG 1**  BTV induces IFN-α/β in afferent skin lymph (A and B) and in serum (C) in vivo. (A and B) Two cannulated sheep were inoculated intradermally with 10^6 TCID50 a-BTV2 (A) and BTV8 (B), respectively. Afferent lymph was collected sequentially, and IFN-α/β was measured in the lymph fluid (U/ml) using a bioassay. (C) Sheep (n = 5) were infected intravenously and subcutaneously with 1 and 5 ml blood, respectively, from a BTV8-infected viremic sheep. IFN-α/β in sera was measured using a bioassay. Significant differences between groups are indicated (*, P < 0.05; unpaired t test).

BTV induces IFN-α/β synthesis by LD lymph and blood cells independently of BTV strain and attenuation. Based on the data obtained as described above (Fig. 1), we hypothesized that lymph and blood pDCs and/or cDCs (the latter being early BTV targets [27]) could be activated by BTV to produce IFN-α/β. As a first approach, we simply enriched lymph and blood cells in cDCs and pDCs by low-density (LD) gradient centrifugation. We found that a rather low dose and wide range of BTV8 TCID50 per cell [(1 to 50) \( \times \) 10^{-2}] was efficient at triggering IFN-α/β synthesis in LD lymph and blood cells (Fig. 2A). The amount of IFN-α/β in the cell supernatant varied among animals (over 30 animals tested). Twenty-six BTV serotypes exist. We tested whether IFN-α/β production in response to BTV was confined to the CD45RB^high^ B^- CD11b^- TCRy/δ^- CD8^- CD11c^- cells (see Fig. S1A to C). Furthermore, we separated the CD45R B^- CD11b^- TCRy/δ^- cells and found to produce IFN-α/β upon exposure to CpG-A (10,000 U/ml), poly(I:C)—a synthetic dsRNA analogue—in Lipofectamine 2000 (120 U/ml), and a-BTV2 (1,000 U/ml). Conversely, CD11c^+^ and B^- CD11c^- CD45RB^- LD lymph cells were found to be unresponsive to CpG-A and Lipofectamine-transfected poly(I:C) (Fig. 3B and not shown). A specific anti-sheep IFN-α ELISA confirmed that BTV induced bona fide IFN-α in sheep lymph pDCs (Fig. 3C). In order to extend these results to blood cells, we purified CD45RB^+^ and CD45RB^- cells from LD PBMCs and confirmed that the IFN-α/β production in response to BTV was confined to the CD45RB^- blood cell fraction (Fig. 3D).

**FIG 2** BTV induces IFN-α/β synthesis in LD skin lymph cells and LD PBMCs in vitro independent of viral strain and attenuation. (A) LD skin lymph cells and LD PBMCs from the same animal (1.5 \( \times \) 10^9/ml) were cultured overnight with increasing doses of BTV8. The results of 1 representative experiment out of 5 are shown. (B) LD PBMCs from 5 sheep (1.5 \( \times \) 10^9/ml) were cultured overnight with BTV strains BTV1, a-BTV2, BTV2, and BTV8 (0.06 TCID50/cell). Culture supernatants were collected, and IFN-α/β was measured using a bioassay (U/ml). Means \( \pm \) SEM are shown. There were no significant differences in IFN-α/β induction between strains (P = 0.6; Kruskal-Wallis test).

pDCs are the main producers of IFN-α/β in lymph and blood cells. We proceeded to identify the cell type that was responsible for the IFN-α/β produced by the LD lymph and blood cells after BTV stimulation. In sheep lymph, cDCs are CD11c^- cells and pDCs are characterized as B^- CD11c^- CD45RB^- cells, which can be isolated using immunomagnetic beads as previously described by us (52). No IFN-α/β could be detected in response to a-BTV2 in the lymph CD11c^- cell fraction, while substantial amounts were found in the CD11c^- cell fraction (500 IU/ml) (Fig. 3A). Lymph pDCs were isolated as described previously (52) and were found to produce IFN-α/β upon exposure to CpG-A (10,000 U/ml), poly(I:C)—a synthetic dsRNA analogue—in Lipofectamine 2000 (120 U/ml), and a-BTV2 (1,000 U/ml). Conversely, CD11c^+^ and B^- CD11c^- CD45RB^- LD lymph cells were found to be unresponsive to CpG-A and Lipofectamine-transfected poly(I:C) (Fig. 3B and not shown). A specific anti-sheep IFN-α ELISA confirmed that BTV induced bona fide IFN-α in sheep lymph pDCs (Fig. 3C). In order to extend these results to blood cells, we purified CD45RB^+^ and CD45RB^- cells from LD PBMCs and confirmed that the IFN-α/β production in response to BTV was confined to the CD45RB^- blood cell fraction (Fig. 3D). We then set up our goal to sort sheep blood pDCs to a high purity level using flow cytometry sorting. As shown in Fig. S1 in the supplemental material, upon CpG-A stimulation, IFN-α/β-producing cells were exclusively found among the CD45R B^- CD11b^- TCRy/δ^- CD8^- CD11c^- cells (see Fig. S1A to C). Furthermore, we separated the CD45R B^- CD11b^- TCRy/δ^-
be replication independent (11). We found that pDCs from FSC\textsuperscript{low} that do not produce IFN-\(\gamma\) high TCF4 RNA levels were also found in fractions 2 and 3 TCF4 (9), much higher than cDCs did (see Fig. S1D). However, pressed high levels of the critical pDC transcriptional regulator primary pDCs, but IFN-\(\gamma\) 30). Rotavirus was found to infect a small fraction of human blood IFN-\(\gamma\)/CpG-A (Fig. 3A and E).

pDCs upon BTV stimulation was lower than that induced by pDCs, we observed that the level of IFN-\(\gamma\) and CD8\textsuperscript{+} CD11c\textsuperscript{−} B cells based on previously published phenotypic studies (3). From cDCs could be sorted to high purity, which allowed us to undergo functional studies. When highly purified sheep blood primary pDCs and cDCs were sorted and stimulated with BTV\textsubscript{8} or CpG-A (10\(\mu\)g/ml), IFN-\(\alpha/\beta\) was measured using a bioassay (U/ml). (B) MACS-sorted CD45RB\textsuperscript{hi} lymph cells (10\(^{6}\) cells/ml) were cultured overnight with a-BTV2 (0.01 TCID\textsubscript{50}/cell), and IFN-\(\alpha\) was measured in cultured supernatant using a ruminant-specific IFN-\(\alpha\) ELISA. (D) MACS-sorted CD45RB\textsuperscript{hi} and CD45RB\textsuperscript{lo} cells from LD PBMCs (1.5 \(\times\) 10\(^{6}\) cells/ml) were cultured overnight with BTV\textsubscript{8} (0.06 TCID\textsubscript{50}/cell) or CpG-A (10\(\mu\)g/ml). IFN-\(\alpha/\beta\) was measured using a bioassay. (E) Blood pDCs (B\textsuperscript{+} CD11b\textsuperscript{+} CD11c\textsuperscript{−} CD45RB\textsuperscript{hi} TCF4\textsuperscript{hi}) were sorted at 98% purity by flow cytometry (2.5 \(\times\) 10\(^{6}\) cells/ml). IFN-\(\alpha/\beta\) was determined in supernatants using a bioassay. Each experiment was repeated at least one or two times, and similar results were obtained. Note that due to the low numbers of purified pDCs that could be collected from 500 ml of blood in most instances, only single experimental pDC points could be done per animal. The results were confirmed by repeating the experiment with several animals.

FIG 3 pDCs from lymph (A to C) and blood (D and E) are the main hematopoietic cell type producing IFN-\(\alpha/\beta\) in response to BTV 

CD8\textsuperscript{−} CD11c\textsuperscript{−} cells into small-, intermediate-, and large-sized cells (fractions 1, 2, and 3, respectively) based on the forward scatter (FSC) profile. As shown in Fig S1C, only fraction 1 (FSC\textsuperscript{high} cells) produced IFN-\(\alpha/\beta\) in response to CpG. Fraction 1 also expressed high levels of the critical pDC transcriptional regulator TCF4 (9), much higher than cDCs did (see Fig. S1D). However, high TCF4 RNA levels were also found in fractions 2 and 3 (FSC\textsuperscript{lo}) that do not produce IFN-\(\alpha/\beta\). These TCF4\textsuperscript{hi} CD45RB\textsuperscript{+} FSC\textsuperscript{hi} cells could correspond to mature pDCs that are smaller than immature pDCs and that do not respond to CpG-A in mice (5). We thus decided to sort “responsive” blood pDCs based on the CD45RB\textsuperscript{hi} B-CD11b\textsuperscript{−} TCR\(\gamma/\delta\) CD8\textsuperscript{−} CD11c\textsuperscript{hi} FSC\textsuperscript{high} phenotype in the subsequent studies. Blood cDCs were sorted as being CD11c\textsuperscript{+} B-CD11b\textsuperscript{−} TCR\(\gamma/\delta\) CD8\textsuperscript{−} CD45RB\textsuperscript{−} FSC\textsuperscript{low} cells based on previously published phenotypic studies (3). From a sheep blood sample (400 ml), between 1 and 5 x 10\(^{6}\) pDCs or cDCs could be sorted to high purity, which allowed us to undertake functional studies. When highly purified sheep blood primary pDCs and cDCs were sorted and stimulated with BTV\textsubscript{8} or CpG-A, only pDCs produced IFN-\(\alpha/\beta\) (Fig. 3E). As for lymph pDCs, we observed that the level of IFN-\(\alpha/\beta\) produced by blood pDCs upon BTV stimulation was lower than that induced by CpG-A (Fig. 3A and E).

BTV infects pDCs but BTV replication is not mandatory for IFN-\(\alpha/\beta\) synthesis in pDCs. IFN-\(\alpha/\beta\) production by pDCs does not usually require viral replication but exceptions do exist (25, 30). Rotavirus was found to infect a small fraction of human blood primary pDCs, but IFN-\(\alpha/\beta\) induction by rotavirus was found to be replication independent (11). We found that pDCs from BTV8-infected LD PBMC cultures (27%) were expressing the nonstructural NS2 protein, indicating that active viral infection occurs in sheep pDCs (Fig. 4A). Furthermore, when highly purified sheep blood pDCs were infected with BTV\textsubscript{8}, accumulation of viral RNA was detected after 48 h of culture (Fig. 4B). We then inactivated BTV\textsubscript{8} using UV irradiation for different periods of time (see Fig. S2 in the supplemental material). After 20 min of UV irradiation of the viral inoculum (UV-BTV), viral replication could not be detected in LD lymph cells that included over 30% cDCs, the primary BTV target for viral replication, and around 2% pDCs (see Fig. S2A). However, IFN-\(\alpha/\beta\) was still found to be produced in the supernatant of LD lymph cells (see Fig. S2B), although at a lower level than with live virus. IFN-\(\alpha/\beta\) synthesis was further decreased with an increased duration of UV exposure, suggesting that UV treatment may possibly alter viral structures important for sensing in pDCs. Viral replication of UV-BTV\textsubscript{8} (20 min of irradiation) could not be detected in BHK21 cells either (data not shown). UV-BTV preparations from 4 strains stimulated LD PBMCs to produce IFN-\(\alpha/\beta\), although at a significantly lower level than live BTV did (see Fig. S2C). Heat-inactivated preparations were inefficient (see Fig. S2C). We confirmed that UV-BTV did not replicate in pDCs from BTV-infected LD PBMC cultures (Fig. 4A) or in the purified pDCs (Fig. 4B). There again, UV-BTV\textsubscript{8} still induced IFN-\(\alpha/\beta\) in highly purified blood pDCs, although to a lower extent than live BTV\textsubscript{8} (Fig. 4C).

BTV is a very difficult virus to purify, and therefore, crude BTV8 preparations were inefficient (see Fig. S2C). We confirmed that UV treatment may possibly alter viral structures important for sensing in pDCs. Viral replication of UV-BTV\textsubscript{8} (20 min of irradiation) could not be detected in BHK21 cells either (data not shown). UV-BTV preparations from 4 strains stimulated LD PBMCs to produce IFN-\(\alpha/\beta\), although at a significantly lower level than live BTV did (see Fig. S2C). Heat-inactivated preparations were inefficient (see Fig. S2C). We confirmed that UV-BTV did not replicate in pDCs from BTV-infected LD PBMC cultures (Fig. 4A) or in the purified pDCs (Fig. 4B). There again, UV-BTV\textsubscript{8} still induced IFN-\(\alpha/\beta\) in highly purified blood pDCs, although to a lower extent than live BTV\textsubscript{8} (Fig. 4C).
and found that purified BTV1 and UV-BTV1 both induced IFN-α/β in highly purified sheep blood pDCs (Fig. 4D), indicating that cell culture contaminants were not responsible for IFN-α/β induction in pDCs. Lymph pDCs were also induced to produce IFN-α/β upon UV-α-BTV2 stimulation. However, as found with LD PBMCs (see Fig. S2C in the supplemental material), heat-inactivated BTV and binary ethyleneimine-inactivated BTV from a commercial vaccine (without adjuvant; data not shown) were not capable of IFN-α/β induction in pDCs, suggesting that specific structural viral motifs need to be preserved to ensure proper sensing for IFN-α/β induction in pDCs.

BTV induces the expression of several cytokine genes in addition to IFN-α, as well as surface CD80/86, in primary sheep blood pDCs. Since pDCs have been shown to produce other cytokines besides IFN-α/β in mice and humans, we determined whether other cytokines, which are likely to have a strong impact on the immune response triggered by the virus, were induced by BTV in sheep pDCs. Indeed, live purified BTV1, UV-BTV1, and crude UV-BTV8 were all found to induce IFN-α, TNF-α, IL-6, and IL-12 gene expression in purified sheep blood pDCs (Fig. 5B and C). UV-BTV8 was also found to increase the expression of the costimulatory CD80/86 molecules on the cell surface of the pDCs from LD PBMC cultures (Fig. 5A, median fluorescence intensity of 66 for unstimulated and 172 for UV-activated pDCs).

IFN-α/β induction by UV-BTV in purified pDCs involves endo-/lysosomal maturation. So far, our data have shown that BTV triggers IFN-α/β secretion, as well as the expression of other cytokine genes and costimulatory molecules, in primary sheep pDCs by a mechanism that does not require viral replication. In order to investigate the mechanism of IFN-α/β induction in pDCs, we used UV-BTV stimulation to avoid possible interference between IFN-α/β synthesis and viral replication. We first addressed whether IFN-α/β induction by UV-BTV required intracellular processing via endo-/lysosomal vesicle maturation. We used bafilomycin A1, an ATPase-specific inhibitor that blocks endosomal and lysosomal acidification. We found that bafilomycin A1 abrogated the IFN-α/β production by LD PBMCs and purified pDCs that were stimulated with either UV-BTV8 or CpG-A (Fig. 6A and B). Chloroquine, which is largely used as an inhibitor of endo-/lysosomal maturation, also prevented the IFN-α/β synthesis induced by UV-BTV in LD PBMCs and in highly purified blood pDCs. However, chloroquine did not affect the IFN-α/β synthesis induced by poly(I:C) delivered with Lipofectamine 2000, which accesses the cytosol without need for endosomal routing (69) (Fig. 6C and D). Cell viability was found to be unaltered by these drug treatments (see Fig. S3A and B in the supplemental material).

IFN-α/β induction by UV-BTV involves TLR7-independent and MyD88-dependent signaling. The inhibition of IFN-α/β induction by inhibitors of endolysosomal maturation suggests that a TLR-mediated signaling may be involved in the IFN-α/β induction by UV-BTV, as is usually the case with pDCs stimulated by viruses. TLR7 is known to mainly sense single-stranded viral RNAs, but it was shown to also respond to specific tertiary dsRNA structures (29). We therefore tested the effect of A151, an oligonucleotide described as a TLR antagonist that inhibits TLR7 and, as expected. However, even at a 50-μg/ml concentration, A151 alone did not inhibit the IFN-α/β induction by UV-BTV (Fig. 7). As A151 strongly inhibited influenza virus-induced IFN-α/β that can signal through TLR7 and -8, this implies that UV-BTV acti-

![Figure 4](https://example.com/figure4.png)
vates IFN-α/β production via a TLR7/8-independent mechanism. Note that bovine pDCs do not express TLR8 mRNA (20).

Although TLR7 did not appear to play any role in the IFN-α/β induction by UV-BTV, we asked whether the MyD88 adaptor molecule could be involved in that process. Indeed, MyD88 not only mediates TLR7 and TLR9 signaling but is also involved in TLR-independent pathways of IFN-α/β induction, such as in the case of the DNA virus sensor DHX36 box helicase in pDCs (36). As RNA interference cannot be used in primary pDCs, in which these molecules induce IFN-α/β by themselves (29), we used a MyD88 homodimerization inhibitor peptide that binds to the MyD88 TIR domain and that is fused to an antennapedia-derived cell permeant motif (43). LD PBMCs from 5 different sheep were cultured overnight with the MyD88 inhibitory peptide or the control peptide and then activated with CpG-A or with UV-BTV for 12 h. Whereas the control peptide, i.e., the antennapedia cell permeant motif, marginally affected the IFN-α/β secretion induced by CpG-A (87% ± 7% [mean ± SEM] of the level in the control) and UV-BTV (83% ± 10% of the level in the control), the MyD88 inhibitory peptide at the 100 nM dose significantly reduced the IFN-α/β production induced both by CpG (28% ± 8% of the level in the control) and BTV (40% ± 6% of the level in the control) (Fig. 8). We could not use the MyD88 inhibitory peptide and its control on purified pDCs for subsequent stimulation by UV-BTV because the sorting procedure compromised the viability of the cells and, thus, the cells did not survive the subsequent preincubation with both the control and the inhibitory peptides. However, the viability of fresh LD PBMCs was not significantly affected by the peptides (see Fig. S3C in the supplemental material). As the only cells capable of IFN-α/β production in LD PBMCs are pDCs (Fig. 3), we can conclude that UV-BTV signaling for IFN-α/β synthesis in pDCs involves, at least in part, the MyD88 adaptor, independent of TLR7/8 activation.

IFN-α/β induction by UV-BTV in pDCs implicates PKR- and JNK-dependent signaling pathways. The cytosolic serine-threonine kinase PKR has been implicated in the IFN-α/β production induced by viruses in many cell types, including cDCs (13, 56), but is not yet studied in pDCs. PKR activation can occur by direct binding of dsRNA or can be induced downstream of other nucleic acid sensors, such as TLR (30) or, possibly, MDA-5 (56, 60). In order to assess the contribution of PKR in the IFN-α/β production induced by UV-BTV, we treated LD PBMC cultures, as well as purified blood pDCs, with 2-aminopurine (2-AP), a commonly used PKR inhibitor (66), or with the oxindole-imidazole C16, a reported selective PKR inhibitor that is efficient in different cell types from different species (2, 21, 24, 31, 33). Because anti-sheep PKR and P-PKR antibodies are not available, we confirmed that C16 used at a 500 nM concentration was potent at preventing PKR phosphorylation induced by poly(I·C) in HeLa cells (data not shown). Both drugs potently inhibited IFN-α/β secretion in LD PBMCs (Fig. 9A and B) while maintaining cell viability (see Fig. S3D and E in the supplemental material). Both

FIG 5 UV-BTV increases CD80/86 surface expression and activates cytokine gene expression in blood pDCs. LD PBMCs were cultured alone (mock) or with UV-BTV (0.06 TCID₅₀/ml). Cells were stained for pDC markers (see Fig. 4A legend) and for CD80/86 surface detection. (A) FACS histogram of CD80/86 and isotype control (ISC) expression on gated pDCs is presented. (B) FACS-sorted pDCs from one sheep (2.5 × 10⁵ cells/ml) were cultured overnight alone or with BTV1 (sucrose gradient purified, 3 μg/ml) or UV-BTV1 (sucrose gradient purified, 3 μg/ml). Cytokine gene expression relative to that of GAPDH was assessed by qRT-PCR, and arbitrary units were calculated by the 2⁻ΔΔCT method. (C) FACS-sorted pDC preparations from 3 different sheep were stimulated overnight by UV-BTV (0.06 TCID₅₀/ml), and cytokine gene expression was evaluated as described for panel B (means ± SEM are shown). *, P < 0.05; paired t test.
drugs also strongly reduced the IFN-α, TNF-α, and IL-12 mRNA expression induced by UV-BTV in purified pDCs (Fig. 9D), whereas they did not affect the CD80/CD86 expression level induced by UV-BTV at the cell surface of pDCs (Fig. 9C).

As PKR was shown to modulate IFN-α mRNA integrity in rotavirus-infected mouse embryonic fibroblasts and in cDCs infected with some specific RNA viruses, we tested whether the oxindole-imidazole C16 treatment would decrease the proportion of polyadenylated versus total IFN-α mRNA induced by UV-BTV. As shown in Fig. S4 in the supplemental material, C16 decreased the IFN-α mRNA amounts detected by random hexamer (total RNA)- and oligo(dT) (polyadenylated RNA)-primed reverse transcription to a similar extent. Thus, blockade of PKR enzymatic activity by C16 does not specifically affect the amounts of polyadenylated IFN-α mRNA in UV-BTV-treated pDCs, suggesting that the PKR-mediated increase of IFN-α mRNA does not involve regulation of IFN-α mRNA polyadenylation.

PKR is known to activate mitogen-activated protein kinases (MAPK), such as stress-activated protein kinase (SAPK)/JNK (22), which has also been implicated in the induction of IFN-α mRNA by cDCs upon adenovirus stimulation (15). LD PBMCs and purified pDCs were cultured with UV-BTV in the presence of SP600125 (a SAPK/JNK inhibitor) (4, 12, 32, 45) and PD184325 (an inhibitor of ERK1/2, which is another member of the MAPK family) (64). We found that UV-BTV IFN-α induction was inhibited by the SAPK/JNK inhibitor by over 60% (Fig. 10B), whereas the ERK1/2 inhibitor had no effect (Fig. 10A). No toxicity was found for the SAPK/JNK inhibitor (see Fig. S3F in the supplemental material).

Altogether, our data show that the induction of IFN-α by UV-BTV in pDCs involves a TLR7/8-independent and MyD88-dependent mechanism. Based on the use of pharmacological inhibitors, they also implicate PKR- and SAPK/JNK-dependent mechanisms.

**DISCUSSION**

Our study reveals novel information concerning the interactions between the dsRNA virus BTV and primary dendritic cell subsets for the induction of IFN-α/β, a key innate immunity cytokine that...
is instrumental for optimal CD8 T cell and antibody responses (39, 41, 51) and for direct antiviral defense. We showed that BTV displays specific interactions with primary pDCs and cDCs: while it infects both cell types, it induces IFN-α/β only in pDCs without requiring viral replication, via a mechanism involving endocytosis and signaling through the MyD88 adaptor without TLR7/8 engagement. Our data show for the first time the involvement of MyD88 in dsRNA virus signaling in pDCs. Thus, MyD88 remains the only known transducing molecule in pDCs for type I IFN induction. However, the evidences presented here from our BTV study demonstrate clearly that viral sensors other than TLR7/8 can be used in pDCs, as was found for TLR9 with some DNA viruses that can alternatively use the DHX36 helicase in pDCs (36).

Although IFN-α/β was detected in blood and lymph early after in vivo BTV infection, it was no longer detected in lymph and blood by day 10. BTV has disappeared in lymph at that time, but relatively high viral loads were still detectable in blood, as previously reported by several authors (49, 53). The lack of IFN-α/β detection in blood at day 10 could be related to the decreased number of pDCs in blood that has been observed in the course of

**FIG 8** Inhibition of MyD88 homodimerization reduces IFN-α/β release by sheep LD PBMCs in response to UV-BTV8 and CpG-A. LD PBMCs from 4 sheep (3 × 10^6 cells/well) were incubated overnight alone or with MyD88 inhibitory peptide or control peptide and then were stimulated for 12 h with UV-BTV8 (0.06 TCID_{50}/ml) (A) and CpG-A (10 μg/ml) (B). IFN-α/β was measured (U/ml) in the supernatants using a bioassay. The IFN-α/β amounts induced by UV-BTV were 16, 31, 63, and 500 IU/ml for the 4 different sheep, respectively. The IFN-α/β amounts induced by CpG-A were 62.5, 125, 250, and 1,000 IU/ml. Data are expressed as the percentages of the IFN-α/β level obtained without peptide treatment. Means ± SEM are shown. *, P < 0.05; paired t test.

**FIG 9** PKR inhibition reduces IFN-α/β release and cytokine gene activation by blood pDCs in response to UV-BTV8. (A, B) 2-AP (A) and C16 (B) were used to block PKR activation in LD PBMCs (empty bars) and FACS-sorted pDCs (filled bars). Drugs were added to cells at indicated concentrations for 30 min before infection, and cells were stimulated overnight with UV-BTV8. IFN-α/β was measured (U/ml) in supernatants using a bioassay. Data are expressed as the percentages of the IFN-α/β level obtained without drug treatment. Data expressed as means ± SEM were obtained with 3 different sheep. *, P < 0.05; paired t test. (C) LD PBMCs were treated or not with C16 (0.5 μM) and were exposed to UV-BTV8 (0.06 TCID_{50}/ml) for 48 h. Cells were stained for pDC and CD80/86 detection by FACS analysis. The histogram represents the medians of fluorescence intensity (MFI) of CD80/86 expression on pDCs. (D) FACS-sorted pDCs were incubated with C16 (0.5 μM) for 30 min prior to UV-BTV8 stimulation and cultured overnight. Cytokine gene expression (IFN-α, TNF, and IL-12p40) was determined by qRT-PCR.
many viral infections, due to selective apoptosis (65). Among other hypotheses, it is also possible that the association of BTV with erythrocytes during viral clearance at day 10 prevents effective interactions between BTV and pDCs (44).

The specific induction of IFN-α/β in pDCs and not in cDCs, even with live virus, could be explained by cell type-specific molecular mechanisms, such as the expression of proper sensors, subcellular trafficking, and translocating cascades. Regarding cell type-specific sensors for IFN-α/β induction by viruses, sensing of Newcastle disease virus in cDCs largely relies on RIG-1 helicase, whereas it relies on TLR7 in pDCs (35). The lack of IFN-α/β synthesis by cDCs that permit replication of BTV may also involve the expression of nonstructural proteins that block IFN-α/β synthesis. Indeed, BTV NS1 and NS2 appear to interfere with the interferon regulatory factor (IRF-3 and -7) response in HeLa cells (62). Following that scenario, the pDC fraction that expresses TLR7/9 inhibitor was not efficient at blocking UV-BTV-induced IFN-α/β, whereas it was efficient at blocking the IFN-α/β production induced by influenza virus. This finding excludes the possibility of both TLR7 and TLR8 involvement in UV-BTV signaling in pDCs.

The lack of TLR7 involvement in IFN-α/β induction in pDCs by an RNA virus as described here is a very rare event. Independence of TLR7 has also been reported for the respiratory syncytial paramyxovirus, which enters and signals via an unknown mechanism in human pDCs after plasma membrane fusion, and not via an endosomal pathway (30). The other known viral sensors in pDCs are DNA sensors, i.e., TLR9 and the DHX36 helicase, which both signal via MyD88. TLR9 is endosomal, while DHX36 is cytosolic. It could be possible that UV-BTV dsRNA reaches the pDC cytosol after uncoating and triggers (a) cytosolic helicase(s) that remain(s) to be identified. The known RIG-1/LGPI-5/LGP2/DDX1 helicases (40, 67, 68, 69) can all bind various forms of dsRNA, and they all signal via MAVS. It could be possible that UV-BTV dsRNA reaches the pDC cytosol after uncoating and triggers (a) cytosolic helicase(s) that remain(s) to be identified. The known RIG-1/LGPI-5/LGP2/DDX1 helicases (40, 67, 68, 69) can all bind various forms of dsRNA, and they all signal via MAVS (also named IPS-1/Cardif/VISA) (59). However, mouse pDCs appear not to rely on MAVS for IFN-α/β induction by viruses (63). Thus, our results suggest the hypothesis that dsRNA sensors, possibly novel helicases linked to MyD88, are implicated in Reoviridae-induced IFN-α/β in pDCs.

PKR is the first dsRNA cell sensor that has been described. Its autophosphorylation and dimerization upon dsRNA binding primarily lead to the establishment of an antiviral state, but it can also trigger IFN-α/β synthesis in different cell types, including cDCs (13), via multiple, complex, and sometimes controversial mechanisms. In human pDCs, the PKR inhibitor 2-AP was found to prevent the IFN-α/β secretion induced by CpG-A (30), indicating that PKR can integrate signal transduction from TLR in this cell type. Some published reports indicate that PKR can control the expression and activation of IRF3 and IRF1, as well as the activation of NF-κB (18, 37, 38, 50), thus having an impact on IFN-α/β gene transcription. In the case of rotavirus-infected embryonic fibroblasts, PKR was shown to promote IFN-α/β secretion not by an increase of transcriptional activity but at the posttranscriptional level via a yet-to-be-defined mechanism (60). Significantly, PKR was recently found to induce IFN-α/β production in coordination with MDA-5-activation by some viruses via the stabilization of IFN-α/β mRNA poly(A) (56). Thus, PKR can promote
IFN-α/β production by many mechanisms, although it has not been studied in pDCs. In the case of BTV in pDCs, PKR activation could be direct, via intracytoplasmic sensing of dsRNA, or indirect, as for TLR signaling. Our results indicate that UV-BTV increases IFN-α/β synthesis and IFN-α mRNA production by a mechanism independent of stabilization of mRNA polyadenylation. Finally, possibly downstream or independently of PKR, we found that signaling via JNK was implicated in IFN-α/β production induced by UV-BTV in primary pDCs, whereas ERK1/2 signaling was not involved. Adenovirus that triggered IFN-α/β production via TLR and MAVS-independent mechanisms in cDCs also used JNK-dependent and ERK1/2-independent signaling related to sensors that were not identified (15).

Altogether, our data show that a dsRNA virus triggers IFN-α/β in primary host pDCs via a novel mechanism that is independent of TLR7/8 but dependent on the MyD88 adaptor. We bring some indications that the PKR and JNK signaling pathways may also be involved. Other members of the Reoviridae family, such as members of the genera Rotavirus and Orbivirus, may use similar pathways, associated with a restriction of IFN-α/β production in pDCs (47).

Our findings aid in the understanding of BTV pathogenicity and, importantly, will have impacts on the improvement of vaccines against the pathogen.

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