Infection of Nonhost Species Dendritic Cells In Vitro with an Attenuated Myxoma Virus Induces Gene Expression That Predicts Its Efficacy as a Vaccine Vector*†

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Recombinant myxoma virus (MYXV) can be produced without a loss of infectivity, and its highly specific host range makes it an ideal vaccine vector candidate, although careful examination of its interaction with the immune system is necessary. Similar to rabbit bone marrow-derived dendritic cells (BM-DCs), ovine dendritic cells can be infected by SG33, a MYXV vaccine strain, and support recombinant antigen expression. The frequency of infected cells in the nonhost was lower and the virus cycle was abortive in these cell types. Among BM-DC subpopulations, Langerhans cell-like DCs were preferentially infected at low multiplicities of infection. Interestingly, ovine BM-DCs remained susceptible to MYXV after maturation, although apoptosis occurred shortly after infection as a function of the virus titer. When gene expression was assessed in infected BM-DC cultures, type I interferon (IFN)-related and inflammatory genes were strongly upregulated. DC gene expression profiles were compared with the profiles produced by other poxviruses in interaction with DCs, but very few commonalities were found, although genes that were previously shown to predict vaccine efficacy were present. Collectively, these data support the idea that MYXV permits efficient priming of adaptive immune responses and should be considered a promising vaccine vector along with other poxviruses.

Recombinant poxviruses are undergoing intensive evaluation as vaccine candidates for a variety of pathogens. Poxviruses are known for their ability to induce strong immunity against their own proteins and against recombinant proteins when genes of interest are introduced into the viral genome (45). Numerous studies involving recombinant viruses have shown that poxvirus infection can induce both T and B cell-dependent immune responses, although poxviruses have developed several strategies to escape host immunity (61). Indeed, several poxvirus vector systems are under study to develop vaccines against infectious diseases (5, 15, 24, 25, 31, 66).

In domestic animals, poxviruses have been shown to be efficient vectors for the production of protective immune responses, notably in rabbits (3), cats (41, 42), horses (27), and ruminants (8, 37, 49, 51). The development of recombinant vaccines for ruminant species should help to implement new vaccine policies and to achieve a distinction between infected and vaccinated animals.

Myxoma virus (MYXV) has already been evaluated as a vaccine vector and is under consideration for use in vaccine development in ruminants (51, 52). Host-restricted MYXV has a number of useful properties as a vaccine candidate, including safety and the ability to incorporate substantial amounts of genetic material for the expression of foreign gene products. We have recently shown that recombinant MYXV is able to infect primary and immortalized ovine cells (52) and that the infection is not productive in this species. Moreover, MYXV-infected ovine cells support the expression of several heterologous proteins (51, 52). In addition, preliminary results demonstrated that sheep injected with MYXV expressing VP60 (the major capsid product of rabbit hemorrhagic disease virus) mount a specific antibody response against the transgene product (51).

Previously published data indicated that MYXV infects dendritic cells (DCs) during natural infection in rabbits, the host species of this virus, suggesting that DCs could be the primary site of MYXV replication (6). Moreover, we recently observed that cells identified as macrophages/dendritic cells, based on their morphology, expressed high levels of MYXV antigens upon intradermal injection of the MYXV SG33 vaccine strain in sheep (52).

DCs are the most potent antigen-presenting cells and play a crucial role during the priming and reactivation of antigen-specific immune responses (2). Following infection with a pathogen, functional changes of DCs are essential because priming and polarization of the immune response depend on these changes (18). Therefore, a better understanding of the modifications in the gene expression of proinflammatory cytokines, chemokines, and stimulatory molecules may prove useful in predicting whether or not a vaccine vector will be effective. Moreover, this understanding may help to identify viral modifications that may improve vaccine efficacy (54).

To increase our understanding of these processes, interactions between ovine bone marrow-derived DCs (BM-DCs) and MYXV SG33 have been studied and the global gene profile of

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DCs in response to MYXV infection was analyzed. Here, we show that MYXV infection induces a strong reprogramming of cells, leading to the expression of proinflammatory cytokines and mobilization of type I interferon (IFN) pathways, cell death, and features associated with the activation of the adaptive immune response.

MATERIALS AND METHODS

Cell lines. Rabbit kidney cells (RK13 and ATCC CCL-17) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Generation and culture of BM-DCs. All animals were maintained in conventional conditions and were euthanized in accordance with local regulations on anesthesia overdose (5 mg/kg of body weight ketamine followed by 15 mg/kg pentobarbital sodium). Protocols for in vitro generation of BM-DCs have been described elsewhere for rabbit (14) and ovine (21) cells. Briefly, bone marrow cells were harvested from the femurs and the sternums of rabbits and sheep, respectively. BM cells were obtained by scraping and were released by crushing the marrow with a syringe plunger in a petri dish filled with Hanks’ balanced salt solution (HBSS) buffer. Cells were passed through a 100-μm nylon mesh to remove small pieces of bone and debris, and red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer. Cells were cultured in bicarbonate-buffered MEME medium (Gibco-BRL) supplemented with 5% FCS was added. After 48 h of incubation at 37°C and 5% CO2, the medium was removed and replaced by solid medium containing Eagle minimum essential medium (MEME) medium (Gibco-BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes (Gibco-BRL), 1% nonessential amino acids, and 50 μM β-mercaptoethanol. The medium was supplemented with 20 ng/ml recombinant ovine and human granulocyte-macrophage colony-stimulating factor (GM-CSF) for ovine and rabbit BM cells, respectively. Fresh medium supplemented with the appropriate cytokine was added every 3 days.

For maturation, DCs were treated with heat-killed Staphylococcus aureus (Pansorbin, 20 μg/ml; Calbiochem) a day before infection as previously described (21).

Isolation of ovine skin DCs. Skin DCs were isolated from ovine skin using a protocol largely based on protocols for human skin DC isolation (65). Briefly, pieces of skin were washed twice in phosphate-buffered saline (PBS), once in 70% ethanol, and once in complete RPMI (eRPMI) with 5% fungizone and 20 μg/ml enrofloxacine (Baytril). The skin was incised to get a lace and incubated in eRPMI with 5 U/ml dispase (Invitrogen) for 1 h at 37°C. The skin pieces were then washed twice in eRPMI and cultured in eRPMI for 2 to 5 days. Cells were harvested, and dendritic cells were stained with Alexa 647-labeled anti-CD11c and incubated with anti-mouse IgG microbeads (Miltenyi Biotec) and separated on MS columns according to the manufacturer’s recommendations.

Viruses. All viruses used in this study were derived from the SG33 attenuated vaccine strain of MYXV (60), modified in our laboratory. The recombinant vaccinia virus p7.5 promoter inserted into the M11L-myxoma growth factor (MGF) locus. Moreover, the SG33-Vp60 virus contains the lacZ gene under the control of the poxvirus late p11 promoter inserted in the same locus.

Viruses were purified by sucrose gradient sedimentation, and titers were determined along with 500 ng of the reference sample to the Agilent 019921 ovine microbeads array slide (Agilent Technologies) according to a reference design without dye swap and following the manufacturer’s protocol. The purity of the enriched fractions was assessed by flow cytometry following A647-labeled anti-CD11c staining on at least 10,000 events.

Apoptosis analysis. For apoptosis analysis, 0.5 × 106 cells (48-well plate) were infected at various MOIs. Sixteen hours postinfection (p.i.), cells were harvested, labeled for cellular markers, and washed in PBS. The cellular concentration was then adjusted to 1 × 106 cells/ml in 1/8 annexin V-binding buffer containing 10 μM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2 and 0.1 μM of PE-labeled annexin V (Alexis) was added to 100 μl of cells (106 cells) and incubated at room temperature for 15 min. The reaction was stopped by the addition of 400 μl of 1/8 annexin V-binding buffer, and the cells were analyzed quickly by flow cytometry.

RNA isolation and microarray hybridization. BM-DCs were harvested after 7 days of differentiation and incubated with 1 μg/ml anti-CD11c monoclonal antibody (MAb) (clone OM1). Alternatively, cell subtypes were separated using an anti-CD11b MAb (clone CC126) and separated on MS columns according to the manufacturer’s recommendations. The purity of the enriched fractions was assessed by flow cytometry following A647-labeled anti-CD11c staining on at least 10,000 events.

Statistical analysis of microarray data. Raw data were normalized with LOWESS using Feature Extraction version 9.5.3.1 and were imported into GeneSpring GX11 software, both from Agilent Technologies. Statistical analysis of microarray data was carried out using one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test for time and a Benjamini-Hochberg false discovery rate control with a threshold q < 0.05. The expression profiles for these genes were clustered over time and entities using the unsupervised hierarchical clustering of GeneSpring based on the Pearson centered gene distance.

Only 8,847 genes on this array were annotated by the Human Ortholog Gene Nomenclature Committee (HGNC) (http://www.sigenae.org/), sheep oligonucle-
otide annotation version 5 of 10 November 2009) (9). As a consequence, only half of our list of differentially expressed (DE) probes was annotated. More information was obtained using the Basic Local Alignment Search Tool program on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ENSEMBL website (http://www.ensembl.org/index.html).

Biological interpretations of the focus genes. Two software programs were used to interpret the lists of focus genes obtained from statistical analysis, Ingenuity Pathway Analysis (IPA) and InnateDB (38). IPA software (version 8.8; Ingenuity Systems) was used to generate biological networks from a list of selected genes and to document the functions of these genes and the canonical pathways in which they are involved.

Gene ontology (GO) analysis and identification of overrepresented pathways and transcription factor binding sites (TFBS) were performed with InnateDB (http://www.innatedb.com).

RT-qPCR. For the quantification of gene expression in infected ovine or rabbit BM-DCs, the RNA samples were assayed by reverse-transcription-quantitative PCR (RT-qPCR). The RT reaction was carried out using a superscript II reverse transcriptase kit (Invitrogen) on 500 ng of RNA. Primers were designed with Primer 3 software (57) using publically available GenBank sequences (see Tables S1 and S2 in the supplemental material). qPCR was performed using specific primer sets for selection of genes with a fold change (FC) of $>2\times\Delta\Delta CT$ and iTaq SYBR green supermix with ROX dye (Bio-Rad) in a final volume of 25 μl using a 7000 real-time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Determination of a stable reference gene was done using the GenNorm version 3.4 Microsoft Excel add-in software package (69). Assays were carried out in duplicate. PCR was performed using the following conditions: denaturation at 95°C for 2 min and 40 amplification cycles consisting of three rounds at 95°C for 15 s and 60°C for 45 s. The results were then analyzed using SDS software version 1.2. Relative gene expression levels were calculated by the threshold cycle ($2^{-\Delta\Delta CT}$) method ($\Delta\Delta CT = CT_{target} - CT_{geometric mean of GAPDH, RPL19, HPRT, S26 genes}$) and $\Delta\Delta CT = \Delta CT_{stimulated} - \Delta CT_{nonstimulated}$ using the gapdh (encoding glyceraldehyde-3-phosphate dehydrogenase), rp19, hprt (encoding hypoxanthine phosphoribosyltransferase), and rps26 genes.

Microarray data accession number. The microarray data are available under accession no. GSE32656 in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/info/linking.html).

RESULTS

SG33 MYXV infects rabbit and ovine dendritic cells but fails to replicate in ovine cells. To show the capacity of MYXV to infect dendritic cells, rabbit and ovine DCs were differentiated in vitro from bone marrow cells in the presence of human or ovine recombinant GM-CSF, respectively. On day 7, GM-CSF DCs were infected with a recombinant virus, SG33-GFP, in which eGFP was under the control of the early/late poxvirus p7.5 promoter. After 16 h of infection, we observed GFP expression in the cells from the rabbits, indicating that host species DCs are highly receptive to MYXV (Fig. 1A). Furthermore, GFP was also detected in ovine BM-DCs (Fig. 1B), albeit at lower levels than observed in rabbit DCs (18% versus
In the ovine cell culture, GFP expression was strictly limited to the CD11c-positive cells (8.17%) and was clearly absent in the CD11c-negative progenitor cells (Fig. 1C), indicating that only differentiated cells had the capacity to support MYXV infection. The MYXV infectivity of ovine DCs and their kinetics of GFP expression were carefully examined (Fig. 1D). At 8 h after infection, the proportion of GFP-positive CD11c-positive cells varied from 5% at an MOI of 0.3 to 20% at an MOI of 3. GFP expression reached a plateau at MOIs above 5, and maximum expression was observed approximately 8 h after infection (data not shown). To evaluate the receptivity of ex vivo DCs, we isolated DCs from the skin of sheep and infected them with SG33-GFP at an MOI of 1 (Fig. 1E and F). After 0, 3, and 8 h of culture, the percentage of GFP-positive CD11c-positive cells varied from 5% at 3 h p.i. to 8% at 8 h p.i. (Fig. 1F), indicating that ovine skin DCs and BM-DCs are equally receptive to MYXV. As the pattern of GFP expression suggested differential receptivity of DCs from host and nonhost species, SG33 replication in rabbit and ovine BM-DCs was compared. Infection of rabbit BM-DCs at an MOI of 0.01, several rounds of replication occurred and MYXV titers increased progressively over time. However, we did not observe an increase of MYXV titer in ovine BM-DCs (Fig. 2A). We also performed a similar experiment in which we infected DCs at an MOI of 1. Infection of rabbit BM-DCs resulted in viral growth curves that closely resembled classical poxvirus growth curves derived from susceptible cell lines, with a continuous increase of MYXV titers until they reached a titer of 6 log, while no increase in MYXV titer was observed in ovine BM-DCs (Fig. 2B). Although the ovine cells are receptive to MYXV infection, we concluded from these analyses that MYXV infection is abortive in ovine BM-DCs.

This incomplete viral cycle may be due to the lack of expression of late genes. To test this hypothesis, ovine BM-DCs were infected with a recombinant MYXV in which lacZ is placed under the control of the p11 late promoter. Rabbit and ovine BM-DCs were infected with recombinant SG33-VP60-LacZ virus, and 24 h p.i., late viral protein expression was evaluated by labeling with X-Gal.
although MYXV infects ovine BM-DCs, replication is defective in this nonhost species.

The Langerhans cell-like DC subset is more receptive to MYXV infection. The culturing of ovine BM progenitors with GM-CSF gives rise to at least two subsets of BM-DCs that can be distinguished on the basis of their CD11b and MHC-II expression (21), monocyte (Mo)-like (CD11b hi MHC-II lo) and Langerhans cell (LC)-like (CD11b int MHC-II hi) DCs. Among these two populations, a higher rate of GFP expression was observed in LC-like DCs than in Mo-like DCs (26.8% versus 10.2%, respectively) (Fig. 3A). The LC-like DC population shares some features with DCs found in the skin. Moreover, we noticed that following overnight culture, the proportion of CD11b int MHC-II hi DCs decreased as the MOI increased (Fig. 3B). The same phenomenon was observed with wild-type SG33, excluding a role for the antiapoptotic M11L gene that was deleted upon GFP insertion. To definitively assess the receptivity of both DC subtypes to MYXV infection and to evaluate the effects of DC maturation, the two DC populations were sorted and then left untreated or treated with maturation stimuli, after which they were infected. After 16 h of incubation, the proportion of CD11b int MHC-II hi DCs decreased as the MOI increased (Fig. 3C, left panel). Furthermore, the proportion of MHC-II hi

FIG. 3. Disappearance of Langerhans-like DCs, a preferred cell target of MYXV following infection. (A) DC subtypes were detected using a CD11b/MHC-II combination, and GFP expression was measured. Data are representative of at least three independent experiments. Ovine BM-DCs were infected with SG33-GFP or SG33 as described in the Fig. 1 legend. Various MOIs of SG33-GFP or SG33 were used to infect BM-DCs. GFP fluorescence was analyzed the next day to quantify the proportion of infected cells. (B) In parallel, the phenotype of recovered cells was analyzed with anti-CD11b and anti-MHC-II (DQ/DR PE) antibodies by flow cytometry. The proportion of CD11b int MHC-II hi cells decreased as the MOI increased. Langerhans cells undergo apoptosis after MYXV infection. (C) Maturation does not abrogate MYXV infection in BM-DCs. BM-DCs were maturated with Sac, as verified by MHC-II upregulation. As indicated above, immature (imDC) or mature (Sac-treated) BM-DCs were infected with SG33 at an MOI of 1. The frequency of GFP-positive cells was assessed 16 h after incubation and was highly comparable between immature and mature BM-DCs. Results are representative of two experiments.
CD11b<sup>int</sup> cells was strongly diminished following overnight culture, suggesting that MYXV induces cell death in these cells irrespective of their maturation state, as observed by the results in Fig. 3B.

To determine whether this cell death is linked to apoptosis induced by MYXV, as reported for other poxviruses, we stained BM-DCs with annexin V that binds to phosphatidylserine on the surface of cells undergoing early apoptosis. Propidium iodide or 7-AAD was also used to detect necrotic cells. Increased annexin V was detected on the surface of GFP-positive DCs, indicating that these cells were undergoing apoptosis 16 h after infection (Fig. 4A). An increase in annexin V binding was also noticed on GFP-negative cells, indicating that a bystander apoptosis of noninfected cells was also occurring. These data indicate that MYXV induces apoptosis not only in a subpopulation of DCs that are more susceptible to MYXV infection but also in neighboring uninfected cells.

**MYXV induces low levels of DC maturation.** In order to investigate the susceptibility of immature and mature ovine BM-DCs to viral cytopathic effects, the maturation of BM-DCs was induced as described above and then cell viability was measured. After infection with SG33-GFP at low (0.3) or high (3) MOIs (Fig. 4B), cells were stained with annexin V and 7-AAD. As previously described, at the same MOI, the percentage of GFP-positive cells was similar between mature and immature BM-DCs. Nevertheless, the maturation state had a strong effect on the susceptibility of BM-DCs to viral cytopathic effects, as a higher level of annexin V and a higher proportion of dead cells (7-AAD<sup>pos</sup>) were observed in mature Sac-treated DCs (30% compared to 10% in immature DCs).

We have shown that maturation does not abrogate the ability of MYXV to infect ovine DCs, as matured cells remained receptive to MYXV. We next evaluated the effect of MYXV infection on BM-DC maturation. For this, BM-DC cultures were infected with various MOIs of SG33-GFP. After 16 h of culture, the levels of costimulatory CD40, CD80, and CD86 molecules were largely unchanged on the surface of both GFP-positive and GFP-negative cells (Fig. 5A). The expression levels of these molecules were very similar to those measured in uninfected cultures, with an increase of less than 10% (Fig. 5B). The upregulation of MHC-II was also minimal following infection. In conclusion, DC maturation does not affect the capacity of MYXV to infect DCs, and MYXV infection alone induces little BM-DC maturation.

**Gene expression profiling of MYXV-infected DCs indicates a strong upregulation of type I IFN-related pathways.** To gain a global view of the gene expression changes induced by MYXV, we infected BM-DCs from three sheep with this virus and performed microarray analysis. Measurement of the RNA integrity number of samples from these sheep indicated lower values (<7.5) for higher MOI conditions (MOI of 3) and later time points. For that reason, transcriptional profiling using an ovine-specific microarray was performed on samples from

![Fig. 4. The maturation state has a strong effect on the susceptibility of BM-DCs to viral cytopathic effects.](http://jvi.asm.org/content/85/21/12987.full.pdf)
early time points (0, 3, and 8 h) at an MOI of 0 or 1. The differential expression of genes over time was assessed for statistical significance with one-way analysis of variance (ANOVA), using a Benjamini-Hochberg false discovery rate of 5% and a fold change (FC) greater than 1.5. This resulted in an annotated list of 390 genes modulated over the 8-h time course, with 233 and 157 genes up- and downregulated, respectively (see Table S3 in the supplemental material). Hierarchical clustering (Pearson centered) allowed the generation of a heat map representation, from which it was evident that infection induced small transcriptome changes during the first 3 h (Fig. 6A and data not shown). In contrast, large changes in gene expression occurred after 8 h. Moreover, when an FC of 5 was applied, a list of 54 genes was obtained, of which all except two were strongly and uniformly upregulated at 8 h (Fig. 6A). Further analysis was focused on data from the 8-h time point.

To confirm these results, RT-qPCR analyses were performed on the same RNA samples, and 28 genes from the list of the most up- or downregulated genes were measured (Fig. 6B, top table). A high correlation was observed between the results of both approaches, with a regression coefficient of r = 0.97, validating the microarray results. Several genes coding for cytokines with T cell-polarizing capabilities were also assessed, and the transcription of genes such as *ifna, il10, il12p35, il12p40, il15*, and *il18* was strongly upregulated following infection (Fig. 6B, bottom table). In Fig. 7, a comparison of the gene expression of rabbit and ovine BM-DCs is shown. These results indicate that genes involved in type I IFN signaling pathways, such as *ccl10, isg15, oas1, mx1, irf3*, and *irf7*, are upregulated in both species. Interestingly, *il10* and *isg15*, respectively, were 7- and 4-fold more highly expressed in ovine than in rabbit DCs, whereas *ccl10* and *oas1* were 22- and 16-fold more highly expressed in rabbit cells.

To gain further insight into the list of 350 genes (*q* value < 0.05 and FC > 2), the Human Genome Organization (HUGO) gene names were imported into Ingenuity Pathway Analysis (IPA) software for gene function analysis. Several functions were statistically affected (Fig. 8A to D). Most systemic functions (Fig. 8A) were related to the humoral immune response and antimicrobial and inflammatory responses. For cellular functions (Fig. 8B), the three most significant functions that were influenced were cell death, cellular growth and proliferation, and cellular development. For cell metabolism (Fig. 8C), carbohydrate metabolism, gene expression, and DNA replication recombination and repair functions were identified, probably related to hijacking of the cellular machinery by viral proteins. The most significantly influenced pathways were type I interferon signaling, p38 mitogen-activated protein kinase (MAPK) signaling, and activation of interferon regulatory factor (IRF) by cytosolic pattern recognition receptors, with *P* values of 4.1 × 10⁻⁶, 7.6 × 10⁻⁵, and 1.3 × 10⁻⁴ and gene ratios of 23.3, 10.3, and 10.8% (Fig. 8D). InnateDB analysis largely confirmed these observations (data not shown). The gene list was separated into up- and downregulated gene lists. For upregulated genes, the significant gene ontology biological
pathway (GO BP) terms were Response to virus, mRNA catabolic process, and Response to IFN-γ. The significant pathways were RIG-I-like receptor signaling pathway, Death receptor signaling, and Extrinsic pathway for apoptosis, confirming the previous results that identified programmed cell death in the IPA analysis (data not shown). For downregulated genes, the information on the pathways that were regulated was more sparse, as protein amino acid N-linked glycosylation and protein polymerization were the only GO BP terms identified.

Transcription factor binding sites (TFBS) for IRF-8 were significantly overrepresented within the list of upregulated genes, and none of these sites was found among the downregulated genes (data not shown).

The transcriptome profile of DC-MYXV interactions is essentially different from other DC-poxvirus interactions. Several reports analyzing interactions of poxviruses with human DCs have previously been published. Hence, our gene profile was compared with the gene profiles induced by the attenuated forms of vaccinia virus (VACV) called MVA and NYVAC (26) and the canarypox virus ALVAC (28) in human DCs. Comparisons were made with a Venn diagram to identify genes in common between the four data sets. Surprisingly, although the analyses revealed some commonalities, the profiles were distinct. The heatmap representation of differentially expressed probes in response to SG33-GFP infection (Fig. 6) indicates the unique expression pattern of MYXV compared to other poxviruses. The figure shows the heat map with a color scale indicating the level of gene expression, with red corresponding to the highest expression and blue to the lowest. The top table lists 28 genes whose differential expression was confirmed by RT-qPCR, and the bottom table are 8 genes encoding major cytokines that were shown to be upregulated.
largely different (Fig. 9A). Indeed, 37, 15, and 22 genes were common between the MYXV and the MVA, NYVAC, or ALVAC gene lists. However, few statistically significant GO BP terms were common between these vectors (see Tables S4, S5, and S6 in the supplemental material). Whereas the genes shared by the MYXV and MVA and NYVAC data sets were essentially related to metabolic functions, MYXV and ALVAC infections induced the upregulation of genes involved in the

FIG. 7. Comparison of differential expression of 11 genes in host species (rabbit) and nonhost species (ovine) by RT-qPCR. Genes involved in type I IFN signaling pathways are upregulated in both species, while ifna and il10 are regulated in different manners.

FIG. 8. Gene ontology analysis of the genes that were differently regulated in response to SG33-GFP. The x axes represent the significance thresholds of given genes involved in a particular function of systemic response (A), cellular function (B), or cell metabolism (C) or in particular pathways (D).
immune response to viruses, including priming of the CD8^+ T cell response (eif2ak2, ifhi, ifitm1, isg15, ifi1, mxi1, mxi2, stat1, and tap1) and virus-induced cell apoptosis (eif2ak2). IRF-7 was central in the network of these genes, as indicated by IPA analysis, and IRF-1-binding sites were statistically overrepresented (P \text{ < } 0.028) within this gene list (Fig. 9B).

DISCUSSION

In this report, we have investigated the response of ovine DCs to MYXV infection as a model of the interaction between a poxvirus and immune cells from a nonhost species. The capacity to undergo maturation and the expression of genes associated with priming of the adaptive immune response have been evaluated.

We showed for the first time that MYXV has several features related to its capacity to serve as a vaccine vector. First, the viral replication cycle was abortive in ovine DCs, reinforcing the notion of the safety of this platform for use in vaccination of sheep. Furthermore, because the lacZ gene driven by a late poxvirus promoter was poorly expressed following MYXV infection of BM-DCs, the recombinant genes should ideally be driven by early promoters. For that reason, the well-known p7.5 promoter is appropriate for vaccine development (45), because it drives GFP expression early after cellular infection and before cell machinery shutdown due to apoptosis.

Second, although the levels of infection achieved at the low MOI are quite high compared to those obtained with other nonreplicative viral vectors, such as adenoviruses (36), MYXV preferentially infects one subtype of DCs. The heterogeneous receptivity of BM-DC subtypes to this virus may be linked to their cell cycle at the time of infection. Langerhans DCs are a main DC population found in the skin (56), and skin DCs were shown to be receptive to infection. Therefore, the intradermal route of vaccine delivery may be the best route of injection to target cells that are receptive to this vector. The mode of virus entry is not well known for MYXV (19). Recently, Sandgren et al. showed that both forms of VACV enter DCs via macrophagocytosis (59). We have previously shown that dextran uptake was more efficient in LC-like DCs, which is a possible explanation for the higher receptivity of these cells to MYXV infection (21).

We have shown that MYXV-infected BM-DCs undergo little maturation based on the upregulation of CD80/86 and CD40 molecules on their cell surface. However, the increased expression of several genes associated with or involved in DC maturation (il18, cd40, ilitm, crebi1, stat1, nfbib, and tnf) indicates that cells are undergoing some maturation due to the infection, which may later be amplified following T cell contact. thbs1 was the most downregulated gene. Thrombospondin-1 (THBS1) is produced not only by DCs but also by platelets, monocytes, and macrophages (43). Binding of THBS1 to CD47 or CD36 prevents the production of proinflammatory cytokines like interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), IL-6, and GM-CSF by maturing DCs without affecting the production of IL-8 and transforming growth factor β (TGF-β); this binding also inhibits DC maturation and antigen presentation to T cells (43). Hence, the downregulation of thbs1 in MYXV-infected DCs should lead to the production of proinflammatory cytokines such as IL-27 or TNF-α that were indeed upregulated in this study. THBS1 is also known to cleave pro-TGF-β into the biologically active form of TGF-β. Repression of THBS1 expression may also limit the activation of TGF-β and preclude the development of associated T helper phenotypes like Th17 or T regulatory cells (34), although the exact role of TGF-β in these differentiation processes is still a matter of debate.

It has been well known that some viruses, such as dengue and influenza viruses, are able to induce DC maturation, which can efficiently prime T lymphocytes (4, 7). Conversely, other
viruses, such as cytomegalovirus (CMV), measles, or HIV, have an immunosuppressive effect as a consequence of disruption of the DC functional activity (23). The effect of poxvirus on DC maturation is a matter of controversy.

Drillien et al. have demonstrated that MVA infection induces the maturation of human DCs, as characterized by the upregulation of costimulatory molecules and the secretion of proinflammatory cytokines (18). This result was confirmed by Liu and colleagues (35). Moreover, it has been shown that infection of immature DCs with the recombinant canarypox HIV vaccine vector induced maturation in an indirect way, mediated by TNF-α (40). Of importance, infected DCs were functional and induced IFN-γ production by CD8 T cells isolated from HIV-infected individuals (40).

However, it has been reported that VACV-infected mouse BM-DCs do not undergo increases in maturation status (71). As a consequence, the VACV-infected DCs are not capable of activating naïve CD8+ T cells. The finding that VACV infection of DCs generates antigen-presenting cells that are incapable of initiating a T cell response is surprising, since a previous report showed direct antigen presentation and the generation of anti-VACV CD8+ T cell responses in mice (62). Another study indicated that MVA was not able to induce maturation of human monocyte-derived DCs (MoDCs), but nevertheless, these cells were still able to present viral antigen to specific human cytotoxic T lymphocytes (CTLs) (30), suggesting that maturation of DCs may happen after interaction with T lymphocytes. UV-treated VACV induces stronger costimulatory molecule expression on mouse BM-DCs than VACV itself, suggesting that active mechanisms inhibit the maturation process (71). In addition to the production of suppressive cytokines (e.g., IL-10) that are known to block DC maturation by orf virus (ORFV) (11, 33), the mechanism used by poxviruses to inhibit DC maturation is still poorly understood. VACV also interferes with the maturation of immature human DCs, as indicated by the inhibition of the expression of many proteins known to be induced during the maturation process (20).

Despite the low levels of DC maturation observed in our experiments, examination of the gene profile of infected cells indicates that nfkβ is significantly upregulated; the activation of NF-κB-dependent pathways leads to maturation (35, 48, 72).

In addition, the upregulation of ifna and ifnb was also confirmed by RT-qPCR. In a model using human myeloid DCs, a culture of unstimulated DCs was found to contain low levels of IFN-α that are able to enhance the maturation and activation of DCs (29).

In our experiments, when mature BM-DCs were infected with MYXV, they were as susceptible as immature DCs; this possibility may overcome the limits described above. The addition of adjuvant molecules to the viral inoculum in order to promote recruitment and maturation of DCs may help to increase the efficacy of MYXV-vectored immunization, as previously described (12, 55). Further work is under way to evaluate this possibility.

Despite low levels of DC maturation, significant changes were detected in the transcription of proinflammatory cytokines, chemokines, and costimulatory molecules, which indicates that MYXV can stimulate the major functions of DCs necessary for the priming of adaptive immune responses. We have demonstrated that MYXV infection induces the upregulation of genes involved in the antiviral response by inducing the type I IFN signaling pathway and its associated genes, including oas1, ifitm1, mxi1, mx2, ifi35, stat1, tap1, ifi1, ifi3, ifi44, and the interferon-inducible isg15. These genes lead to viral degradation and, thus, inhibit viral replication (22, 53). However, IFN production is probably not the mechanism that precludes viral replication in ovine DC cultures, although isg15 was more highly expressed in ovine cells than in rabbit cells. We have also shown that late gene expression is defective in nonhost species cells. The binding of IFN-α to its receptor stimulates the intracellular IFN signaling cascade and induces an antiviral response by inducing genes involved in OAS1, PKR, and RIG-I-like receptor signaling (63). Of the various MAPK pathways, the p38 signaling cascade has the most important role in the generation of IFN-mediated signals.

Depending on the nature and timing of the maturation stimulus, DCs can preferentially induce different T helper cell responses (16, 32). Our study indicates that infection with MYXV induces the transcription of proinflammatory cytokines, such as ifna, il6, il12, il27, and cxcl10, which are involved in the major DC functions, including communication between innate and adaptive immune cells and the polarization of the adaptive immune response to the Th1 phenotype. CXCL10, the most upregulated gene in response to MYXV infection in both the microarray analysis and RT-qPCR, is a chemotactic factor for T cells and activated NK cells (13), and its regulation has been suggested to occur via IRF-3 (28). CXCL10 is expressed within tissues following viral infection, suggesting an important role for this chemokine in host defense by contributing to lymphocyte activation, extravasation, and accumulation of virus-specific T cells at the site of infection (58). In our experiments, few cytokines were upregulated, including il27, il12p40, il18, and il6. IL-27, an immunomodulatory cytokine, can both promote the early stages of a Th1 response by inducing the proliferation of naïve CD4 T cells (50) and limit the Th2 response (1). IL-12p40 and IL-18 both have an effect on Th1 response by inducing the production of IL-2 and IFN-γ; IL-12p40 in turn promotes IL-18 expression (46). IL-6, another upregulated cytokine, is known to stimulate the humoral response by activating B lymphocytes. Altogether, the upregulation of these genes indicates that the response of DC to MYXV infection is notably oriented toward the Th1 pattern of response, which is further confirmed by the efficient induction of inflammatory and antibody responses that was reported by Pignon et al. (51, 52). Similarly, the other poxviruses, MVA, NYVAC, and ALVAC, induce the transcription of proinflammatory cytokines, such as ifna, il2, cxcl10, and il6.

During the course of infection, transcriptional profiling of MYXV-infected BM-DCs showed that the total RNA amount and RNA quality decrease upon infection, indicating an active process of RNA degradation; this has also been observed in MVA, NYVAC, and ALVAC infection. For VACV and ALVAC infections, this result may be explained by the activation of 5′-oligoadenylate synthetase-like messenger (OASL) that is responsible for RNA degradation. However, other mechanisms are also present, because this enzyme was found not to be significantly induced in our study, although RNA breakdown was observed. This RNA degradation may be linked to the apoptosis that was clearly seen following infection.
It was clear from our studies that LC-like DCs die rapidly by apoptosis upon MYXV infection. Indeed, apoptosis was more profound in the LC-like DCs than in Mo-like DCs. The development of apoptosis was also confirmed by the gene profiling study, where a group of genes involved in cell death signaling, including daxx, fas, casp5, casp8, irf1, irf8, il1b, and cd40, were upregulated. IL-18 is known to upregulate the FasL-mediated cytotoxicity of murine natural killer cells (67, 68) and to be part of a complex regulatory circuit involved in the causation of apoptotic cell death. The expression of Fas is upregulated by IFN-γ (70), which is in turn induced by IL-18. Apoptotic death has also been observed in other pox-DC interactions studied to date. For ALVAC infection, at an MOI of between 0.2 and 1, apoptosis was not induced until 24 h postinfection. Under our conditions, at an MOI of 1, apoptosis was already ongoing only 8 h after infection.

The role of DC apoptosis in the immune response is still unclear, but antigens presented by apoptotic cells are reported to increase immunogenicity and are likely to be more effectively processed for cross-priming (17). Indeed, antigens released from dying cells can be taken up via endocytosis and guided to alternative pathways of antigen presentation. The delayed apoptosis observed in our study may be due to the production of apoptosis inhibitors (64). SERP2 and M11L are both lacking in the SG33-GFP genome. The deletion of the M11L gene in the recombinant SG33-GFP virus was not associated with enhanced apoptosis, although its role in the inhibition of apoptosis in MYXV-infected RL-5 rabbit T cells has previously been described (39). The few DCs that were still primeable up to 8 h after MYXV infection may be sufficient for T cell priming. Indeed, DC migration to the lymph node was evident 6 h after footpad injection in mouse models (47). In the lymph node, resident DCs may take up MYXV-infected apoptotic cells and present epitopes to T cells.

These data indicate that MYXV may be an effective vaccine vector in nonhost species, such as ovines. Comparison of the gene profiles induced by other poxviruses with that induced by MYXV indicated that some regulated genes were common, notably genes belonging to the type I IFN and inflammatory responses. Interestingly, these genes are also present in a list of genes induced by the live attenuated yellow fever vaccine in humans, a vaccine which is probably the most effective vaccine (YF17D) ever developed (44). This observation suggests that some genes involved in the inflammatory and IFN responses may be predictive of protective immune responses and may be used as a benchmark to evaluate and improve the efficacy of novel vaccines.

In conclusion, microarray profiling of a poxvirus-DC interaction helps delineate interesting features of a vector candidate and paves the way for additional improvements to a promising platform.

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REFERENCES


