Expression of interleukin-1 beta and interleukin-6 in white-tailed deer infected with Epizootic haemorrhagic disease virus

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Cytokine,
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White-tailed deer.

Summary
The pathogenesis of Epizootic haemorrhagic disease (EHD) in white-tailed deer (*Odocoileus virginianus*, WTD) may be related to factors other than direct viral damage caused by replication in endothelium, such as the release of cytokines. This study focused on interleukin-1 β (IL-1) and interleukin-6 (IL-6), which have been shown to be variably upregulated in Bluetongue virus (BTV) infected cattle and sheep endothelial cultures possibly explaining species susceptibility to BTV. We evaluated circulating and tissue levels of IL-1 and IL-6 in WTD experimentally infected with EHD virus serotype 2 (EHDV-2). Circulating levels of IL-1 were assayed by ELISA. RT-PCR and immunohistochemistry (IHC) were used to detect upregulation of IL-1 and IL-6 mRNA as well as protein expression, respectively. RT-PCR was also used to determine whether IL-1 and IL-6 were upregulated in WTD peripheral blood mononuclear cells (PBMC) infected with EHDV-2 *in vitro*. We found increased circulating levels of IL-1 and upregulation of IL-1 mRNA and protein expression and upregulation of IL-6 mRNA in tissues of WTD infected with EHDV. Upregulation of mRNA levels of IL-1 and IL-6 in EHDV infected PBMCs was also observed. Findings suggest a role for IL-1 and IL-6 in the pathogenesis of EHD in WTD.

Espressione di interleuchina-1 beta e interleuchina-6 nel cervo dalla coda bianca (Odocoileus virginianus) infetto dal virus della malattia emorragica epizootica

Parole chiave
Cervo dalla coda bianca,
Citochina,
Interleuchina-1 β (IL-1),
Interleuchina-6 (IL-6),
Virus della Malattia emorragica epizootica.

Riassunto
La patogenesi della Malattia emorragica epizootica (EHD) nel cervo dalla coda bianca (*Odocoileus virginianus*) oltre all'azione diretta del virus conseguente alla sua replicazione nell'endotelio, può essere associata al rilascio delle citochine. Il lavoro è incentrato sullo studio dell'interleuchina-1 β (IL-1) e interleuchina-6 (IL-6) che, in colture di cellule endoteliali di bovini e ovini infettate con il virus della Bluetongue (BTV), hanno una concentrazione diversa di IL-1 e IL-6 suggerendo una suscettibilità diversa al BTV legata alla specie. Nel corso di questo studio sono stati valutati i livelli circolanti e tissutali di IL-1 e IL-6 in cervi dalla coda bianca sperimentalmente infettati con il sierotipo 2 del virus dell'EHD (EHDV-2). I livelli circolanti di IL-1 sono stati misurati con ELISA. Le concentrazioni di IL-1 e IL-6 mRNA e l'espressione della proteina sono state rilevate, rispettivamente, con RT-PCR e immunoistochimica (IHC). La metodica RT-PCR è stata impiegata anche per rilevare la concentrazione di IL-1 e IL-6 nelle cellule mononucleate del sangue periferico di cervi dalla coda bianca infettati *in vitro* con EHDV-2. Lo studio ha permesso di riscontrare un aumento dei livelli circolanti di IL-1, della concentrazione di IL-1 mRNA e dell'espressione della proteina e della concentrazione di IL-6 mRNA nei tessuti degli stessi animali infetti. Si è inoltre osservato un aumento della concentrazione IL-1 e IL-6 nei PBMCs infettati con EHDV. I risultati suggeriscono l'importanza dell'IL-1 e dell'IL-6 nella patogenesi dell'EHD nei cervi dalla coda bianca.

Epizootic haemorrhagic disease (EHD) is an important viral disease of white-tailed deer (WTD) (*Odocoileus virginianus*) caused by Epizootic haemorrhagic disease virus (EHDV) serotypes 1, 2, or 6 (*Reoviridae: Orbivirus*) and vectored by biting midges in the genus *Culicoides* (Sellers 1991, Allison *et al.* 2010). As EHD viruses are both monocytotropic and endotheliotropic (Tsai and Karstad 1973, Stallknecht *et al.* 1997), microvascular damage seen with their infection may represent individual or combined effects of viral infection of endothelial cells and inflammatory mediators, such as interleukin-1 β (IL-1) and interleukin-6 (IL-6), released from infected cells. Interleukin-1 β is produced by macrophages, endothelium, keratinocytes, epithelium, and cells of the central nervous system (CSN) (Dinarello 1991). It can activate endothelium, increasing the procoagulant activity of vessels and causing vasodilation, or inducing acute-phase responses, *e.g.* fever, which are typical of EHD (Dinarello 1991, Quist *et al.* 1997). Interleukin-1 β can also induce IL-6 production by macrophages and endothelial cells in response to tissue injury and other inflammatory stimuli (Abbas *et al.* 2000). Interleukin-6 may induce either a pro- or an inhibitory inflammatory response and is one of the principal mediators of the clinical manifestations of tissue injury, including fever, cachexia, leucocytosis, thrombocytosis, increased plasma levels of acute phase proteins, and decreased plasma levels of albumin (Wilder 1998).

Transcription of genes encoding IL-1 and IL-6 has been shown to be upregulated in microvascular endothelial cell (MEC) cultures of both sheep and cattle infected with Bluetongue virus (BTV) (DeMaula *et al.* 2001, DeMaula *et al.* 2002, Drew *et al.* 2010). In cattle MEC cultures, transcription of both cytokines was greatly increased and expression potentiated by the addition of inflammatory mediators released from BTV infected MEC cultures; whereas in sheep MEC cultures, transcription of IL-1 was greatly increased, while IL-6 was only minimally induced and the addition of inflammatory mediators suppressed the production of both (DeMaula *et al.* 2001, DeMaula *et al.* 2002). Similar to species-specific differences between cattle (resistant) and sheep (susceptible) to BTV, WTD from 2 subspecies, *Odocoileus virginianus texanus* from Texas and *Odocoileus virginianus borealis* from Pennsylvania, have been reported to vary in susceptibility to EHDV (Gaydos *et al.* 2002). Despite deer in both subspecies developing similar levels of viraemia and humoral immune responses, severity of disease and mortality was restricted to the *O. v. borealis* group, suggesting that variable expression of disease was due to innate resistance.

In this study, we hypothesised that variable induction of IL-1 and IL-6 and the response of endothelium to these cytokines could help explain the variability in severity of EHD in 'susceptible' and 'resistant' deer populations. The goal was to characterise the pathogenesis of EHDV-2 in WTD, specifically looking at the expression of IL-1 and IL-6 in deer from susceptible and resistant populations. More specifically, the study aimed at determining whether tissue and circulating levels of IL-1 and IL-6 were upregulated in EHDV-2 infected WTD; and assess whether IL-1 and IL-6 upregulation occurs in WTD monocytes infected with EHDV-2 *in vitro*.

Seven 6-month-old WTD fawns of potentially varying susceptibility, as described in experimental (Gaydos *et al.* 2002) and field studies (Flacke*et al.* 2004), were chosen: *Odocoileus virginianus macaourus* (Eastern Kansas) and *O. v. borealis* (New Jersey) fawns were considered as 'susceptible', while *O. v. texanus* (Western Kansas) fawns as 'resistant' for data analysis. All animals were managed in accordance with the applicable USDA Animal Welfare Regulations and the Guide for the Care and Use of Laboratory Animals (National Research Council Committee 2011). Five deer (3 from New Jersey and 2 from Western Kansas) were inoculated with EHDV-2 [$10^{7.03}$ 50% tissue culture infectious dose ($TCID_{50}$)]; 2 deer from Eastern Kansas received sham inoculation and served as controls. One infected deer died on day 7 post inoculation (PI); the remaining surviving animals were euthanized on day 8 PI. Animals were monitored for clinical signs, blood was collected for haematology, coagulation assays, and ELISA, and samples from skin and buccal mucosa were collected at necropsy for histopathology, IHC, real time polymerase chain reaction (RT-PCR), and viral isolation. Clinical disease severity scores (CDSS) were calculated for infected deer as previously described (Gaydos *et al.* 2002).

The deer used for *in vivo* infection were divided into groups: control deer (n = 2), 'susceptible' (n

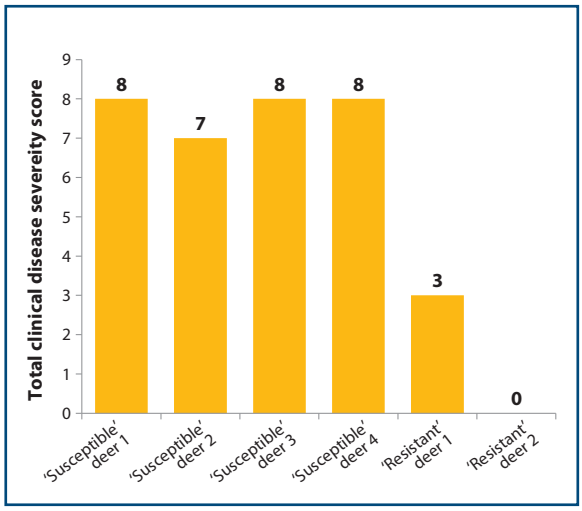

Figure 1. *Total clinical disease severity scores (CDSS) for 'susceptible' (n = 4) and 'resistant' (n = 2) fawns infected with serotype 2 of Epizootic haemorrhagic disease virus.*

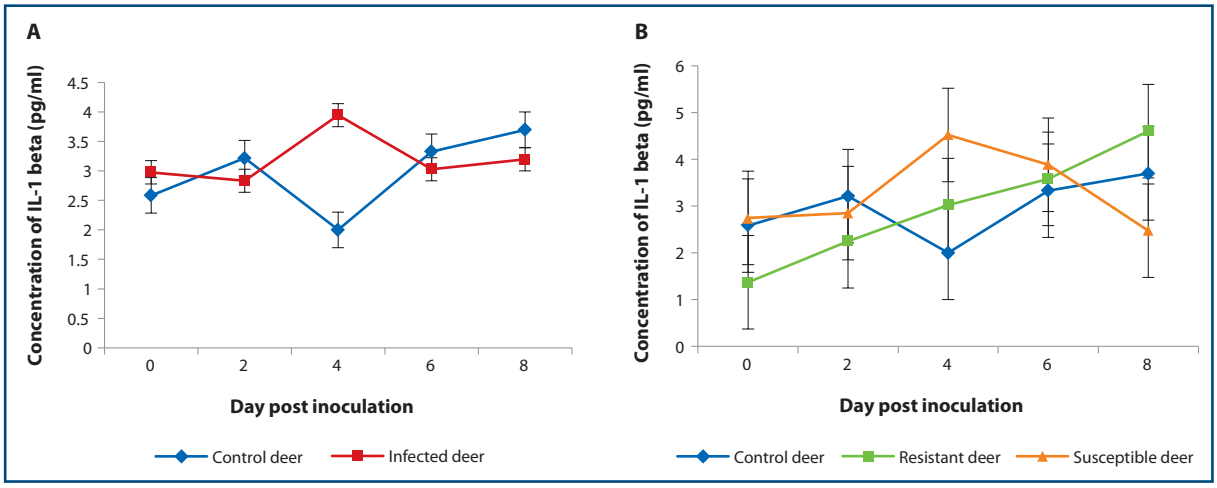

Figure 2. *ELISA for plasma levels of IL-1 on control and white-tailed deer (WTD) infected with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2).* (**A**) Average concentration of IL-1 (pg/ml) in control (n = 2) and all infected deer (n = 6) over time in the experiment. (**B**) Average concentration of IL-1 (pg/ml) in control (n = 2) and EHDV-2 infected WTD of 'susceptible' (n = 4) and 'resistant' (n = 2) populations.

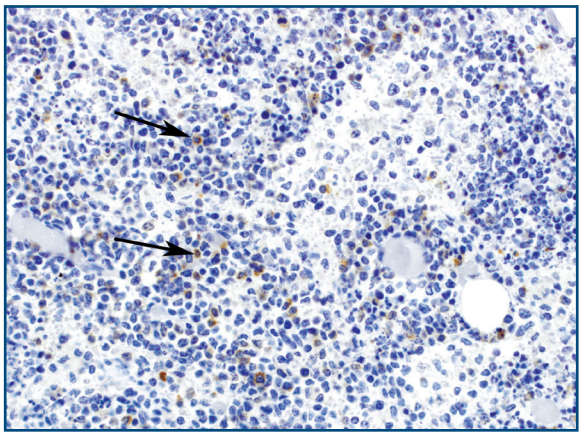

Figure 3. *Immunohistochemistry for IL-1 done on right prescapular lymph node from a 'susceptible' white-tailed deer infected with serotype 2 of the Epizootic haemorrhagic disease virus (NJ4), sampled at necropsy (day 8, PI).* Magnification x400. Arrows point to macrophages and lymphocytes expressing IL-1 protein.

= 4), and 'resistant' (n = 2) EHDV infected deer. Components of the CDSS (*e.g.,* haematology) were analysed by analysis of variance and significant differences were compared by t-test (Graph Pad software, San Diego, CA). The ELISA and RT-PCR results from the control, 'susceptible' and 'resistant' EHDV infected deer (as mentioned previously) were analysed by assessing the variance and significant differences were further examined using student's t-test. $P < 0.05$ was considered to be statistically significant.

Clinical disease severity scores (CDSS) in the deer from the potentially resistant population were lower than those from potentially susceptible populations (Figure 1). Plasma from day 0, 2, 4 6 and 8 PI was used to assay circulating IL-1 levels (human IL-1 beta Quantikine kit, R&D Systems). The mean level was higher in all infected deer, peaked early in infection (day 4 PI) as compared to the uninfected deer, and this increase was more marked in 'susceptible' deer with greater CDSS, when deer were separated into 'resistant' and 'susceptible' deer groups (Figure 2A, Figure 2B).

Deer became viraemic by day 4 PI, blood viral titers were highest in both groups of deer on day 6 PI, and peak titers were higher in 'susceptible' ($10^{5.5}$ $TCID_{50}$) than in 'resistant' deer ($10^{4.5}$ $TCID_{50}$). Virus was first detected in buccal mucosa and skin on day 4 PI and titers were higher in 'susceptible' (buccal mucosa = $10^{3.5}$ $TCID_{50}$; skin = $10^{3.25}$ $TCID_{50}$) deer than in 'resistant' (buccal mucosa = 10^{2} $TCID_{50}$; skin = 10^{2} $TCID_{50}$) deer.

At necropsy, higher viral titers were observed in the tissue samples (especially in spleen and lung) of 'susceptible' deer (spleen = $10^{4.18}$ $TCID_{50}$; lung = $10^{4.095}$ $TCID_{50}$) compared to the 'resistant' deer (spleen = $10^{3.76}$ $TCID_{50}$; lung = $10^{2.93}$ $TCID_{50}$). Additionally, deer with high CDSS had higher blood titer ($10^{2.93\text{-}7.2}$ $TCID_{50}$ on day 8 PI) than those with low CDSS (blood titer: $10^{3.93\text{-}5.1}$ $TCID_{50}$ on day 8 PI).

Changes at necropsy were severe in 'susceptible' deer and insignificant in the 'resistant' deer. Gross lesions in the 'susceptible' deer included oedema of the head, neck and lungs, multifocal haemorrhage and congestion in heart, ruminal papillae, and serosa of abomasum and intestines. Histologically, mucosal haemorrhage in the rumen, abomasum, and intestines and oral mucosal ulceration, petechial haemorrhage and congestion were observed. Formalin-fixed, right and left prescapular lymph nodes, buccal mucosa, rumen, abomasum, lung, spleen, and intestines taken at necropsy and buccal mucosa biopsies from days 0, 2, 4, 6, and 8 PI were stained for IL-1 by immunohistochemistry using mouse anti-ovine IL-1 antibody (Serotec, Raleigh,

North Carolina, US 1:50) and an avidin-biotin detection system with DAB as chromogen. Rare lymphocytes and macrophages stained positively in right or left prescapular lymph nodes of the control deer. There was greater IL-1 protein expression (in the lymphocytes and macrophages) in lymph nodes of 'susceptible' infected deer as compared to the 'resistant' deer especially in the lymph nodes draining the site of inoculation (right prescapular lymph nodes) (Figure 3).

Semiquantitative RT-PCR for IL-1 and IL-6 was done on prescapular lymph node taken at necropsy and buccal mucosa taken on day 0, 2, 4, and 6 PI using primers: IL-1-forward primer: 5'-GCTGAGGAACAGTGCC and IL-1-reverse primer: 5'-CCAGCACCAGGGATTT; IL-6-forward primer: 5'-ATTCAATCAGGAGACTTGCT and IL-6-reverse primer: 5'- CGGTTGGAGTGGTTACTAGA. Actin was used as a housekeeping gene (Keefe *et al.* 1997). Specific PCR products for IL-1 and IL-6 were 181 bp and 185 bp, respectively. Actin was 212 bp. The mean band intensity was expressed as relative intensity units and the ratio between the mean band intensity of the sample IL-1 or IL-6 and beta-actin was calculated, with beta-actin coexpression considered 100%, to normalize for initial variations in sample concentration and as a control for reaction efficiency.

In right prescapular lymph node, mRNA expression of IL-1 was higher in the infected deer than in the control deer, but it was not statistically significant ($P = 0.3870$). IL-1 mRNA expression in 'susceptible' deer was higher than 'resistant' or control deer. The average relative intensities for IL-6 mRNA were higher in the infected deer as compared to the uninfected deer (Figure 4 A; $P = 0.0185$). Expression of IL-6 mRNA was greater in 'susceptible' infected deer than in the 'resistant' infected ($P < 0.0001$) and control deer (Figure 4B; $P = 0.0058$).

To assess the production of IL-1 and IL-6 in WTD peripheral blood mononuclear cells (PBMCs) infected with EHDV-2, semiquantitative RT-PCR was performed. White-tailed deer PBMCs were inoculated with EHDV-2 (multiplicity of 0.02 $TCID_{50}$/cell) and bacterial lipopolysaccharide at 20 ng/ml (Sigma, St. Louis, MO, USA), and media were positive and negative controls, respectively. About 0.1 µg RNA (using RNA-Bee, Amsbio, Cambridge, MA, USA) extracted from adherent and non-adherent cells, harvested at 0 hours, 24 hours, and 48 hours PI, was used in the RT-PCR. Upregulation of both IL-1 and IL-6 mRNA was seen in the PBMCs inoculated with EHDV-2 at 24 hours PI and expression of both was also greater than controls at 48 hours PI (Figure 5).

Combined roles of IL-1 and IL-6 have been investigated in disease processes as indicators of outcome or as linked to severity of clinical signs in several viral infections. Results of our *in vivo* studies indicate that IL-6 and IL-1 may play a similar role in EHD. Upregulation of IL-1 and IL-6 was greater in deer with high CDSS, suggesting that these cytokines may contribute to the severity of clinical disease. IL-1 and IL-6 in tissue and circulation of infected WTD may also act as an indicator of the outcome of EHD with increased levels signifying a poorer prognosis.

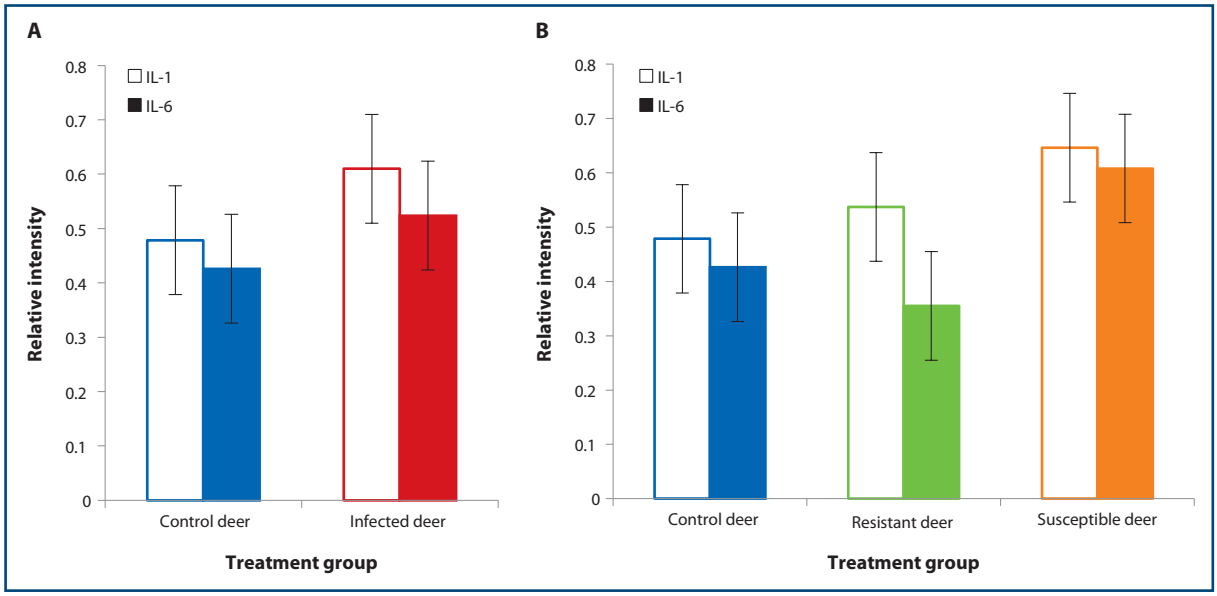

Figure 4. *Semiquantitative RT-PCR for IL-1 and IL-6 done on right prescapular lymph nodes (side of inoculation) taken at necropsy from mock-infected and white-tailed deer infected with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2).* (**A**) Relative intensities for IL-1 and IL-6 expression in control and EHDV-2-infected WTD (control deer n = 2; infected deer n = 6); and (**B**) control and EHDV-2-infected deer from potential resistant and susceptible populations (control deer n = 2; resistant deer n = 2; susceptible deer n = 4).

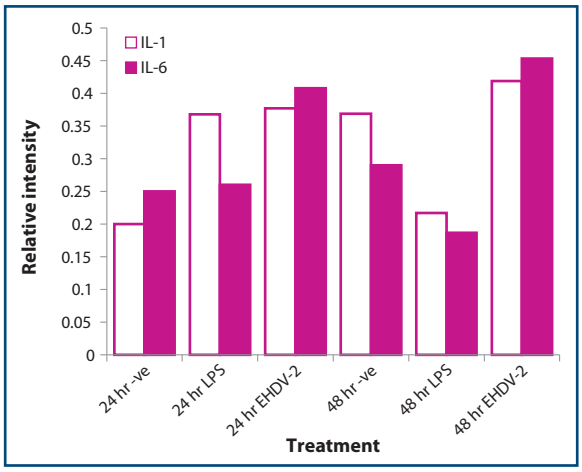

Figure 5. *RT-PCR for IL-1 and IL-6, done on peripheral blood mononuclear cells isolated from a white-tailed deer in an* in vitro *study.* Relative intensities for IL-1 and IL-6 expression (deer n = 1). EHDV-2 = serotype 2 of the Epizootic haemorrhagic disease virus.

The deer used in this study were from 3 populations and potentially different subspecies and could potentially be similar to the 'resistant' and 'susceptible' deer studied by Gaydos and colleagues (Gaydos *et al.* 2002). Indeed, results of our *in vivo* infection suggested that the severity of disease was greater in deer from the suspected 'susceptible' subspecies as compared to the suspected 'resistant' subspecies. Though inoculated with the same viral dose, in general, higher viral titers were observed in blood and the tissue samples from the suspected 'susceptible' deer as compared to the 'resistant' deer suggesting that decreased ('resistant' deer) or increased ('susceptible' deer) viral replication may contribute to the variation in the severity of disease associated with infection in these 2 subspecies.

Immunohistochemistry for IL-1 indicated that upregulation of protein expression was primarily limited to lymph nodes and was greater in lymph nodes draining the site of inoculation than elsewhere in the body. Transcription of genes encoding for IL-1 and IL-6 was up-regulated in lymph nodes and, like protein expression, mRNA expression of IL-1, was greater on the side of inoculation. There was no apparent upregulation of transcription of genes encoding IL-1 and IL-6 in the buccal mucosa, a common tissue with lesions, during infection. Absence of IL-1 and IL-6 mRNA upregulation in buccal mucosa even in the presence of virus in the tissue suggests that upregulation of IL-1 and IL-6 at the tissue level is not totally dependent on viral replication. It is possible that the presence of lesser number of monocytes and lymphocytes in buccal mucosa, as compared to the lymph node, could also explain lower levels of IL-1 and IL-6 in buccal mucosa, but other cells can produce these cytokines.

A haemorrhagic disease in humans caused by Marburg and Ebola viruses, which is quite similar to EHD in WTD, results in the activation of monocytes, triggering the release of IL-1 and IL-6 contributing to the clinical signs like coagulation disorders and variable degrees of haemorrhage (Ströher *et al.* 2001). Viral infection has been known to trigger cytokine production in BTV infected ovine monocytes (Dhanasekaran *et al.* 2013, Drew *et al.* 2010), which in turn has been suggested to contribute to the pathology associated with Bluetongue in sheep.

The results of our semi-quantitative RT-PCR on infected PBMCs also suggest that EHDV infection of mononuclear cells *in vivo* could trigger the release of IL-6 and possibly IL-1 contributing to the severity of haemorrhagic disturbances associated with EHD in WTD.

Our *in vivo* and *in vitro* studies indicate that IL-6 and IL-1 may play a role in the pathology associated with Epizootic haemorrhagic disease in WTD and could be used as indicators of severity or outcome of the disease. However, the number of animals used in this study was small, warranting the need for further studies with more deer from potentially 'susceptible' and 'resistant' populations to clarify the role of IL-1 and IL-6 and other cytokines in the pathogenesis of EHD in WTD, especially with regards to variation in expression of the disease.

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