Lymphotropism and host responses during acute wild-type canine distemper virus infections in a highly susceptible natural host

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The mechanisms behind the in vivo virulence of immunosuppressive wild-type morbillivirus infections are still not fully understood. To investigate lymphotropism and host responses, we have selected the natural host model of canine distemper virus (CDV) infection in mink. This model displays multisystemic infection, similar to measles virus and rinderpest virus infections in their susceptible natural hosts. The wild-type CDVs investigated provoked marked virulence differences, inducing mild versus marked to severe acute disease. The mildly virulent wild-type virus induced transient lymphopenia, despite the development of massive infection of peripheral blood mononuclear cells (PBMCs) exceeding that determined for the highly virulent wild-type virus, indicating an inverse relationship between acute virulence and the extent of viraemia in the investigated wild-type viruses. Single-cell cytokine production in PBMCs was investigated throughout the acute infections. We observed Th1- and Th2-type cytokine responses beginning in the prodromal phase, and late inflammatory responses were shared between the wild-type infections.

INTRODUCTION

Morbilliviruses are lymphotrophic and induce multisystemic infections in their highly susceptible natural hosts, causing disease with high morbidity and mortality worldwide. Members of the genus Morbillivirus within the family Paramyxoviridae include Measles virus (MV) of primates, Canine distemper virus (CDV) of carnivores and Rinderpest virus (RPV) of cloven-hoofed animals. Natural infections by all of these share common features, including lymphopenia and inhibition of lymphocyte proliferation in the acute phase (Griffin, 2007; Heaney et al., 2002; von Messling et al., 2003; Yanagi et al., 2006). Although live vaccines have effectively reduced the incidence of disease, the highly contagious morbilliviruses are still a major problem in human and veterinary medicine (Greene & Appel, 2006; Rima & Duprex, 2006).

Like MV and RPV, CDV causes a typical acute infection characterized by virus replication in cells of the lymphoid system, with subsequent spread to tissues throughout the body. Acute clinical morbillivirus disease often takes a fatal course in highly susceptible natural hosts such as young dogs, mink and ferrets due to the highly multisystemic virulence, which includes central nervous system (CNS) involvement and profound immunosuppression, favouring opportunistic secondary pathogens (Appel, 1969; Blixenkrone-Møller, 1989; Kauffman et al., 1982; von Messling et al., 2006). Despite the clinically relevant immunosuppression, another hallmark of virulent morbillivirus infections is the induction of efficient and lifelong humoral and cellular immunity in hosts that eventually survive and appear to clear the virus (Appel et al., 1982; Griffin, 2007).

The molecular events behind morbillivirus-induced perturbation of the immune system have been studied extensively and, based on in vitro studies, several mechanisms have been proposed to be involved (Heaney et al., 2002; Schneider-Schaulies & Dittmer, 2006; Yanagi et al., 2006). Thus, accumulating evidence strongly links immunosuppression and lymphopenia not only to direct effects following infection and subsequent destruction of signalling lymphocytic activating molecule (SLAM)-positive immune cells and precursor cells in the acute phase of the infections, but also to indirect effects modulating the host innate and adaptive immune systems (Cruz et al., 2006; Devaux et al., 2008; Heaney et al., 2003; Karp et al., 1996; Schlender et al., 1996; Tatsuo et al., 2001; von Messling et al., 2006). The relative impact on virulence of the different mechanisms modulating the immune system in vivo is, however, not yet clarified.

The morbillivirus genome encodes two accessory products, the V and C proteins. In recent infection studies with different recombinant CDVs, including attenuated V- or...
C-defective viruses, cytokine mRNA-expression profiles were analysed in leukocytes of inoculated ferrets (Svitek & von Messling, 2007; von Messling et al., 2006). Early cytokine expression was observed in animals selected for later survival of infection, whereas the corresponding expression in lethally infected individuals was reported to be largely undetectable. The latter studies were targeted to the first week of infection, yet investigation of individual animals over time through the acute phase of wild-type CDV infections including the clinical phase could reveal possible subsequent modulation of cytokine responses.

Here, we assessed infection of peripheral blood mononuclear cells (PBMCs) and host responses over time through the acute phase, including the clinical period, of wild-type CDV infections in mink. The natural virus–host model in mink was selected because this and other mustelid species appear to be highly susceptible to wild-type CDV infections (Crook et al., 1958; Pearson & Gorham, 1987; von Messling et al., 2003).

We have previously assessed the acute and high virulence of the Snyder Hill wild-type CDV in mink (Blixenkrone-Møller, 1989; Dahl et al., 2004). Its lymphotropism and multisystemic spread to epithelia and the CNS have been mostly studied in dogs (Appel, 1969; Krakowka & Koestner, 1977; Summers et al., 1984). Although CDV is monotypic in serological terms, like MV, genetic differences exist and the Snyder Hill wild-type virus belongs to a major European phylogenetic lineage of circulating CDV genotypes, whereas the Snyder Hill genotype belongs to a major American lineage (Martella et al., 2006).

The two wild-type isolates investigated in the present study provoked subclinical versus marked to fatal acute clinical disease in mink. Our results show transient lymphopenia for the mildly virulent wild-type virus, despite the development of massive viraemia exceeding that deter- mined for the highly virulent wild-type virus. Furthermore, distinct Th1 and Th2 cytokine responses, beginning in the prodromal phase, and late-onset inflammatory responses were determined.

**METHODS**

**Viruses.** The DK91 wild-type strain originates from dog organs stored at −80 °C of an acute distemper-diseased pup (Blixenkrone-Møller et al., 1993; Bolt et al., 1997). The Snyder Hill wild-type virus investigated in the present study, representing a North American wild-type CDV, also originates from a distemper-diseased dog (Gillespie et al., 1958). Its high virulence was demonstrated by in vivo studies in dogs (Appel, 1969). The Snyder Hill wild-type isolate used in the present study has been propagated by successive inoculation of organ homogenates in dogs (Appel, 1969; Appel et al., 1982) and maintained in frozen tissues. A dog-tissue homogenate of the Snyder Hill wild-type virus was further propagated by in vivo passages in mink (Blixenkrone-Møller, 1989; Dahl et al., 2004).

The inoculation materials of the two wild-type strains used in the present study were 10 or 20 % (w/v) lung and spleen homogenates in RPMI 1640 medium (Gibco Life Technologies), cleared by centrifugation at 1500 g for 10 min and frozen in aliquots at −80 °C. The Vero cell-adapted Onderstepoort strain of CDV was used for virus neutralizing-antibody tests.

**Infections in mink and assessment of clinical signs of virulence.** Twenty-two CDV-seronegative female wild-type mink (*Mustela vison*), 10–11 months of age, were purchased from Østergård Farm (Roskilde, Denmark). The mink had no records of Aulerian mink disease virus or mink enteritis virus. All experimental procedures were in accordance with the requirements of the Danish Animal Care and Ethics Committee. First, to isolate the DK91 wild-type CDV in vivo, a pooled lung and spleen organ homogenate from an acute distemper-diseased pup (Blixenkrone-Møller et al., 1993) was inoculated into two young CDV-naive mink. One week post-inoculation (p.i.), the two mink developed viraemia with a high degree of virus antigen-positive PBMCs. The two infected mink were euthanized 1 week p.i. A pooled organ homogenate from these mink, infected with the CDV wild-type strain designated DK91, was used to infect two animals (second passage in mink). These animals developed high-degree cell-associated viraemia 1 week p.i., as in the first passage in mink. Preliminary data indicated that the DK91 wild-type infection was associated with mild suppression of the lymphocyte proliferative response in vitro (data not shown).

Thereafter, in group A, five mink were inoculated with 1.0 × 10⁶ TCID₅₀ ml⁻¹ of the CDV Snyder Hill wild-type strain, which had been passed in vivo in mink like the DK91 wild-type strain as described above, and five mink were mock-infected. In group B, four mink were inoculated with 1.6 × 10⁶ TCID₅₀ ml⁻¹ of the DK91 wild-type strain (first mink passage) and four mock-infected mink served as negative controls. To secure comparable and massive challenge doses, intraperitoneal and mucosal routes were used for inoculation.

Under anaesthesia, 4 ml 20 % (w/v) challenge virus suspension was injected intraperitoneally. An additional 1 ml virus suspension (10 %, w/v) was dripped into each conjunctiva and the nostrils. The mink were anaesthetized by intramuscular administration of ketamine hydrochloride (15 mg kg⁻¹) and xylazine hydrochloride (1 mg kg⁻¹) (Ketaminol Vet. and Narcoxyl Vet.; Intervet). The mink were monitored at least twice daily for clinical signs of virulent infections, including anorexia, depression, dermatitis, conjunctivitis, rhinitis, coughing, dyspnoea, diarrhoea and CNS symptoms. Individuals developing marked signs of distemper disease were anaesthetized prior to euthanasia with pentobarbiturate. At the end of the experiment (group A, 31 days p.i.; group B, 56 days p.i.), the remaining animals were euthanized. The experiments were performed in March–April and May–August in the same year, respectively. Peripheral blood for flow cytometry was collected from the tail vein in heparinized capillary tubes. Blood samples for serum collection and heparin-stabilized blood samples for purification of PBMCs were drawn by puncture of the vena cephalica accessoria. A Ficoll density gradient (Ficoll Paque; Amersham Biosciences) was used for purification of PBMCs as described previously (Blixenkrone-Møller et al., 1992). Tissue samples collected post-mortem were stored at −80 °C until testing for virus RNA and antigen content.

**Assessment of virus infection in PBMC and organ systems.** Intracellular CDV antigen staining of PBMCs and tissue smears was carried out as described previously (Blixenkrone-Møller et al., 1992). Briefly, cells and tissue smears from the lymphoid, respiratory and urinary tract systems were acetone-fixed on glass slides and incubated with a mAb against the CDV nucleoprotein (N) (Orvell et al., 1985) or normal mouse serum (negative controls) (code no. X0910 DakoCytomation). Fluorescein isothiocyanate (FITC)-labelled rabbit F(ab')₂ antibody to mouse immunoglobulins (code no. P0313;
For detection of virus infection in the CNS, tissue RNA extractions and a two-step RT-PCR assay were performed. Total RNAs were isolated from 80–100 mg homogenized brain tissue by use of RNA Now according to the supplier (Ozyme, Biogentex). cDNA was synthesized from 1 µg total RNA by using reverse transcriptase and random priming with hexamers (Advantage RT-for-PCR kit; Clontech). The reactions were incubated at 95 °C for 1 min and 42 °C for 60 min. For PCR, Taq DNA polymerase (Clontech) was used; the PCRs were incubated at 95 °C for 1 min and amplification was carried out by 35 cycles of 95 °C for 30 s, 64 °C for 1 min and finally 64 °C for 1 min. A 430 bp sequence in the CDV phosphoprotein (P) gene was amplified by using the ‘universal’ primer pair first described by Barrett et al. (1993). PCR amplification with a primer pair for the cellular glyceraldehyde-3-phosphate dehydrogenase gene, yielding a 568 bp product, served as a control for the RNA extractions (Dahl et al., 2004). The sensitivity of the RT-PCR assay for the Snyder Hill and DK91 wild-type viruses was determined in virus-infected Vero cells expressing dog SLAM (Vero/dSLAM cells) (Seki et al., 2003). The limit of detection of the two-step RT-PCR was in the range 0.03–0.10 TCID50 in 1 µg total RNA.

Characterization of antibody response in mink. CDV neutralizing-antibody titres were determined in Vero cell cultures by using a TCID50 format microtitre assay as described previously (Blixenkrone-Møller et al., 1994) with a few modifications. Briefly, serial twofold dilutions of heat-inactivated serum starting at 1 : 10 were added to approximately 50 TCID50 of the standard virus and tested in triplicate. A known positive mink serum was used as positive control and the virus dilution without serum served as negative control. The titres were calculated by using the method of Reed & Muench (1938).

Flow-cytometric analysis of leukocytes and PBMC subpopulations. Five microlitres of heparin-stabilized blood was lysed for 6 min in 0.2 ml lysis solution (0.15 M NH4Cl, 10 mM KHCO3, 1 mM disodium EDTA, pH 7.3), after which 0.2 ml PBS was added. Subsequently, 5 × 104 cells were analysed by fluorescence-activated cell sorting (FACS) on a FACS Calibur flow cytometer (Becton Dickinson). Populations of granulocytes, lymphocytes and monocytes were gated based on their light-scatter characteristics in a forward scatter versus side scatter diagram as described previously (Jensen et al., 2003). For determining absolute counts of leukocytes, the instrument particle (cell or latex bead) registration efficiency was measured by using BD TruCount tubes (Becton Dickinson) (Harslund et al., 2007). The staining methods for subpopulations of mink PBMCs using cell-surface markers have been described previously (Castelruiz et al., 2005; Chen et al., 1997; Jensen et al., 2003). Briefly, 350–400 µl heparinized blood was lysed for 6 min in 11 ml lysis solution, after which 2 ml PBS was added. The cell suspension was centrifuged for 5 min at 800 g and washed once in PBS. For staining of markers (CD3, CD8, CD79 and CD14), cells were washed in 1 % normal mink serum/PBS and then incubated on ice for 20 min, followed by washing in 1 % normal mink serum/PBS and then incubated on ice for 45 min. For staining of markers (CD3, CD8, CD79 and CD14), cells were washed in 1 % normal mink serum/PBS and then incubated on ice for 20 min, followed by washing in 1 % normal mink serum/PBS and then incubated on ice for 45 min. For staining of markers (CD3, CD8, CD79 and CD14), cells were washed in 1 % normal mink serum/PBS and then incubated on ice for 20 min, followed by washing in 1 % normal mink serum/PBS and then incubated on ice for 45 min. For staining of markers (CD3, CD8, CD79 and CD14), cells were washed in 1 % normal mink serum/PBS and then incubated on ice for 20 min, followed by washing in 1 % normal mink serum/PBS and then incubated on ice for 45 min.

Single-cell cytokine production in PBMCs by flow cytometry. For intracellular cytokine staining, blood cells were harvested as described above and stimulated with 20 ng phorbol-12-myristate-13-acetate ml−1 (PMA; Sigma) and 1 µg ionomycin ml−1 (Sigma) in RPMI 1640 medium supplemented with 10 % fetal calf serum (both from Gibco Life Technologies), 10 µg brefeldin A ml−1 (Sigma) and antibiotics (200 units penicillin ml−1 and 200 µg streptomycin ml−1). The identification of mink cross-reactive mAbs against cytokines and staining methods for mink PBMCs have been described previously (Pedersen et al., 2002). Briefly, the cells were cultured for 4 h at 37 °C and in 5 % CO2, washed and treated with 4 % paraformaldehyde in PBS. The intracellular stainings were performed in the presence of 0.1 % saponin (Sigma) and mAbs against tumour necrosis factor alpha (TNF-α), IL-6, IL-4, IL-10, IL-2 (clone 609; Egan et al., 1994), and IL-18 (clone 2G8; Serotec) were added to the cells. The second-layer FITC-conjugated antibody was as described above.

With a lymphocyte/monocyte gate, we collected 2 × 104 cells for analysis and the percentage of cytokine-positive cells was calculated after subtraction of eventual positive signals from the isotype-matched IgG control preparations (code no. X0931 and X0943; DakoCytomation).

Characterization of viraemia by flow-cytometry analysis. Leukocytes were harvested and prepared for intracellular immuno-sensing as described above. A mAb against the CDV N was used as the primary antibody (Örvell et al., 1985), and the second-layer FITC-conjugated antibody was as described above. With a lymphocyte/monocyte gate, we collected 2 × 104 cells for analysis.

Statistical analyses. Data are presented as means ± SEM. Student’s t-test was used for statistical evaluations. All P-values were two-tailed and were considered statistically significant when the associated probability was <0.05.

RESULTS

Experimental infections with two wild-type strains of CDV in mink: marked strain-related differences suggesting an inverse relationship between acute clinical disease and the extent of viraemia

The DK91 wild-type CDV originates from an acutely and fatally infected dog and was isolated in vivo in mink in the present study. The pathogenic properties of the Snyder Hill wild-type virus have so far been mostly studied in dogs (Appel, 1969; Krakowka & Koestner, 1977) and, due to its acute and high virulence, this virus has been used as a challenge strain in vaccine trials (Appel et al., 1984; Dahl et al., 2004; Krakowka & Koestner, 1977; Stephensen et al., 1997).

To compare the virulence properties and the cytokine profiles and host responses elicited over time through the acute infection phase, five mink were challenged with the Snyder Hill and four mink with the DK91 wild-type strain, while nine mock-inoculated mink served as controls. We monitored the presence of viraemia for up to 4 weeks and observed lymphocyte-associated viraemia persisting from weeks 1 to 3/4 p.i. in the virus-inoculated mink, as summarized in Table 1. Moreover, both the Snyder Hill and the DK91 wild-type viruses spread from the lymphoid system to other organ systems. Notably, in the group of Snyder Hill-infected mink, a high degree of multisystemic spread was determined post-mortem at weeks 3 and 4 p.i. (Table 1).
The five mink challenged with the Snyder Hill virus showed a prodromal phase with transient signs of disease on days 8–11 p.i. in the form of reduced activity and food intake, which in all mink proceeded to signs of distemper disease with one or more typical clinical signs including depression, conjunctivitis, diarrhoea, CNS symptoms and/or acute fatality (four of five mink exhibited severe disease and/or acute fatality). One mink died on day 21 p.i. and another on day 24 p.i.; two mink showing severe clinical disease were euthanized (on days 28 and 31 p.i.). Due to the highly virulent course of the infections, the Snyder Hill experiment (group A) was terminated 31 days p.i.

The DK91 wild-type experiment (group B) ended 56 days p.i. Clinical signs of disease were not observed.

### Table 1. Comparison of virulence of two wild-type strains of CDV in mink

Data are expressed as number of mink that tested positive/number of mink tested or with box symbols as detailed below. Each box represents an individual animal.

<table>
<thead>
<tr>
<th>Mink group</th>
<th>Clinical signs of distemper</th>
<th>Lymphopenia</th>
<th>CDV antigen</th>
<th>CDV RNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week after inoculation</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>Snyder Hill</td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>0/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>DK91</td>
<td>0/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>0/4</td>
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</table>

*Five mink were challenged with the Snyder Hill wild-type (group A) and four with the DK91 wild-type (group B) of CDV. The mock-inoculated mink served as controls.

†Filled boxes, lymphocyte count <500 lymphocytes (μl blood)^−1 and/or >4-fold reduction in lymphocyte count compared with the pre-inoculation counts (day 0 p.i.); shaded boxes, >2.2-fold reduction; empty boxes, ≤2.2-fold reduction.

‡The presence of intracellular CDV nucleoprotein (N) antigen was analysed by immunofluorescence assay on acetone-fixed PBMCs at weeks 1–4 p.i., and post-mortem on tissue sections of the spleen, lung and urinary bladder epithelium. In addition (cf. Fig. 2), virus N-antigen in PBMCs was analysed by flow cytometry in DK91-infected mink. Filled boxes, ≥20% positive PBMCs; shaded boxes, >5% positive PBMCs; hatched boxes, ≤5% positive PBMCs; empty boxes, viraemia not detected.

§Spread of the infection to brain tissues was detected by amplification of CDV phosphoprotein (P) gene in a two-step RT-PCR.

¶Marked clinical disease appeared from day 19 p.i. All mink showed marked clinical signs of canine distemper, with one or more symptoms including depression, conjunctivitis, diarrhoea, CNS symptoms and/or acute fatality (four of five mink exhibited severe disease and/or acute fatality). One mink died on day 21 p.i. and another on day 24 p.i.; two mink showing severe clinical disease were euthanized (on days 28 and 31 p.i.). Due to the highly virulent course of the infections, the Snyder Hill experiment (group A) was terminated 31 days p.i.

¶¶The DK91 wild-type experiment (group B) ended 56 days p.i. Clinical signs of disease were not observed.

The five mink challenged with the Snyder Hill virus showed a prodromal phase with transient signs of disease on days 8–11 p.i. in the form of reduced activity and food intake, which in all mink proceeded to signs of distemper disease with one or more typical clinical signs including depression, conjunctivitis, diarrhoea, CNS symptoms and/or acute fatality (Table 1). Monitoring of infection-related body-temperature rises was not successful by use of standard thermometers, as the handling of the animals induced unpredictable fluctuations. Marked clinical disease appeared from day 19, and mink developing severe signs were euthanized. We terminated the Snyder Hill experiment on day 31 p.i., due to the observed high clinical virulence. In contrast, all DK91-challenged mink had subclinical courses (Table 1). Moreover, the results suggest an inverse relationship between acute clinical disease and the degree of viraemia in the investigated wild-type viruses (Table 1).

**Solid adaptive immune response in the face of high-grade lymphocyte-associated viraemia**

Cell-associated viraemia was analysed by flow cytometry in the DK91 wild-type virus-infected mink (Fig. 1). Based on gating in the forward scatter versus side scatter dot plots, the vast majority of the infected PBMCs were lymphocytes. Thus, the number of antigen-positive cells peaked on day 6 p.i., with up to 74% positive lymphocytes. For the monocyte population, 14% were found to be antigen-positive on day 6 p.i. (Fig. 1). We did not carry out similar flow-cytometry measurements for the Snyder Hill-infected mink, as the limited number of cells available (due to the profound lymphopenia) were prioritized for cytokine measurements.

To assess the quality of the adaptive immune responses elicited by the two wild-type viruses, we measured CDV neutralizing-antibody production. By 2 weeks p.i., all mink

![Fig. 1. Cell-associated viraemia detected by FACS analysis of the percentage of CDV antigen-positive PBMCs from mink infected with the DK91 wild-type virus (n=4). Quantifications of virus-positive cells were performed on gated lymphocyte and monocyte populations. Percentages of virus-positive lymphocytes (●) and monocytes (○) are shown in separate curves. In mock-inoculated animals, virus antigen was not detected (data not shown).](image-url)
infected with the DK91 wild-type virus mounted strong neutralizing-antibody responses, reaching titres around 100 [mean log_{10}(titre), 2.2], whereas the antibody production appeared with slow kinetics in the Snyder Hill-infected mink, two of which never mounted detectable responses before fatal disease outcome (Fig. 2).

**Transient lymphopenia despite massive viraemia in mildly virulent wild-type infections**

The Snyder Hill challenge provoked drastic lymphopenia, which persisted until the end of the evaluation 3 weeks p.i. (Table 1). In contrast, lymphocyte counts in the DK91-challenged mink dropped only transiently, despite the demonstrated massive infection of the circulating lymphocytes (Table 1).

We further investigated the kinetics of the populations of circulating T lymphocytes (CD3^+ cells, including the CD8^+ subpopulation), B lymphocytes (CD79^+ cells) and cells of the monocytic lineage (CD14^+ cells) in the DK91-challenged mink. A slight perturbation in the relative proportions of the investigated cell populations appeared at the onset of lymphopenia 1 week p.i., indicating that the T cells were affected by the depletion to a higher extent than the B cells and cells of the monocyte lineage. Thus, the percentages of T lymphocytes and of the CD8^+ subpopulation decreased (Fig.

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**Fig. 2.** Virus neutralizing-antibody responses in mink infected with the DK91 (◇, n=4) or Snyder Hill (●; n=5, except for weeks 3 and 4 p.i., where n=3) wild-type strains. All mock-inoculated mink tested negative for neutralizing antibodies in serum during the experiments (data not shown). The horizontal dashed line indicates log_{10}=2, which equals a neutralizing titre of 100.

**Fig. 3.** FACS analysis of the percentage of (a) CD3^+ , (b) CD8^+ and (c) CD79^+ lymphocytes and (d) CD14^+ monocytes in PBMCs from mink inoculated with the DK91 wild-type strain (●, n=4) or from mock-inoculated mink (◇, n=4).
3a, b), whereas those of B lymphocytes peaked during the early phase of the lymphopenia, followed by a moderate drop in the later phase (Fig. 3c).

The demonstrated early rises in B-cell and monocyte-lineage percentages (Fig. 3c, d) appeared to reflect the early-onset T-cell depletion in this mildly virulent wild-type infection. Further studies, including data from double staining of the fraction of virus-positive cells for each cell population, may provide more insight into the marked drop in the number of CD79+ and CD14+ cells between days 7 and 10 p.i. The decrease in the B-cell percentages at later time points (Fig. 3c) could indicate that these cells probably succumb less readily to CDV infection than T cells.

Systemic cytokine response during the acute phase of wild-type CDV infections

To assess cytokine profiles over time through the acute phase of the two wild-type infections, we investigated peripheral blood leukocytes by flow cytometry. The production of a major mediator of the innate and inflammatory responses (TNF-α) and the humoral (IL-4) and cellular (IFN-γ) immunity were assessed.

Leukocytes were stimulated with PMA and ionomycin to obtain sufficient sensitivity in the measurement of mink cytokines at the single-cell level by flow cytometry as described previously (Aasted et al., 2002; Jensen et al., 2003), and the frequencies of cytokine-producing PBMCs assessed in CDV-infected mink were compared with those of mock-infected mink (Fig. 4).

We found TNF-α- and IL-4-producing cells almost exclusively among lymphocytes. Cells positive for IFN-γ were distributed primarily in the lymphocyte gate, and the slight baseline production (5 %) detected in the monocyte gate did not change significantly during the experiments (data not shown).

Fig. 4(a) shows the ratios of PBMCs producing the proinflammatory early-response cytokine TNF-α in the virus-inoculated mink and in the mock-inoculated control groups on the corresponding days. In the first 10 days p.i., the two groups of wild-type-infected mink did not appear to mount any statistically significant changes in the ratios of TNF-α-producing cells, whereas late-onset TNF-α responses started in weeks 2–3 p.i. Solid responses of >2-fold increases in the two groups of mink that received wild-type viruses compared with the control groups did not occur until week 3 p.i. (Fig. 4a).

Fig. 4(b, c) shows the production of a hallmark Th1 cytokine, IFN-γ, and the Th2 cytokine IL-4. After week 1, increases in the percentages of IFN-γ-producing lymphocytes appeared in the Snyder Hill-challenged as well as in the DK91-challenged group of mink, compared with the results for the control groups. As for the TNF-α responses, >2-fold increases in the percentage of IFN-γ-producing cells and IL-4-producing cells compared with the control groups were not demonstrated until week 3 p.i. for either wild-type infection (Fig. 4b, c).

DISCUSSION

The DK91 wild-type virus showed a mildly virulent course through the acute infection phase in our natural host model, in contrast to the acute, severe disease outcome characteristic of the Snyder Hill wild-type infection (Appel, 1969; Appel et al., 1982; Dahl et al., 2004), allowing the assessment of lymphotropism and cellular responses elicited by wild-type strains with profound inherent differences in virulence. These insights may prove relevant for morbilliviruses in general.

The relative importance of factors involved in the CDV-associated development of immunosuppression is not well-defined. However, the rapid invasion of lymphocytes by
CDV has been found to be linked closely to SLAM recognition and constitutes a virulence determinant (Condack et al., 2007; Tatsuo et al., 2001; von Messling et al., 2006). We found a rapid and unexpectedly extensive virus invasion of lymphocytes in the mildly virulent wild-type infections. The demonstrated evasion of extensive lymphopenia in the face of swift virus invasion of lymphocytes is probably multifactorial. A possible interpretation of these results is that, for the mildly virulent wild-type infections, a reduction occurs in the proportions of virus-infected lymphocytes and precursor cells undergoing rapid destruction compared with those of the highly virulent wild-type infections.

Our results show that mink infected with the mildly virulent DK91 wild-type virus recovered progressively from the induced lymphopenia, whereas the Snyder Hill-infected mink developed irreversible lymphopenia.

Our present results regarding undetectable cytokine responses in PBMCs during the first 7 days of infection with wild-type CDV in mink support recent studies targeting days 3 and 7 of CDV infections in ferrets (Svitek & von Messling, 2007; von Messling et al., 2006). Those studies reported largely on shutdown of early-response cytokine expression in ferrets incubating lethal CDV infections, whereas infections with attenuated recombinant CDVs with defects in the V reading frame did not suppress early cytokine responses. The latter study based on blood leukocytes further identified early cytokine expression in animals within the first 7 days of eventually non-lethal infections (Svitek & von Messling, 2007). Also, analogous experiments with MV in monkeys have shown that wild-type viruses appear to control the early innate and antiviral responses in studies focusing on PBMCs during weeks 1 and 2 after infection (Devaux et al., 2008).

This disengagement of the host innate immune system has been shown to be associated with the morbillivirus V protein, which interferes with STAT protein and cytokine signal transduction and is required to control the inflammatory and IFN responses in vivo (Devaux et al., 2008; Nakatsu et al., 2008; von Messling et al., 2006). Also, the C protein appears to be involved via different actions (Fontana et al., 2008a; Nakatsu et al., 2008; Takeuchi et al., 2005).

Reports based on naturally occurring distemper have so far mainly been on cases in dogs, and cytokine expression was measured on a single time point in the final moribund phase. Among the 14 cases investigated, dogs with viraemia and severe systemic disease were reported with low or absent cytokine mRNA induction in analysis of whole blood (Gröne et al., 1998).

Importantly, our data presented here, being the first to assess the kinetics of systemic cytokine responses in PBMCs throughout the acute phase of CDV infections, suggest that the apparent virus-mediated control of the early systemic inflammatory response was alleviated gradually, beginning after the peak of the viraemic phase in weeks 2–3 p.i. of wild-type infections in mink.

Although the measurements on stimulated PBMCs can be argued to be artificial, it appeared that the set-up using relative comparison of the percentages of cytokine-producing cells in infected versus mock-inoculated groups is also an advantage when taking into account the outbred background of the host animals. The sole purpose of stimulation is to create sufficient sensitivity of measurements at the single-cell level by flow cytometry (Aasted et al., 2002; Castelruiz et al., 2005; Jensen et al., 2003).

We demonstrated increases in the ratios of IFN-γ-producing lymphocytes starting after week 1 for both wild-type infections, compared with the results for the control groups. IFN-γ provides an important innate defence in response to virus infection and cooperates with dendritic cells and T lymphocytes in the generation of adaptive cellular responses (Fontana et al., 2008b; Goodbourn et al., 2000).

Our results on the relatively slow kinetics of the systemic IL-4 responses did not appear to correspond closely with the early solid neutralizing-antibody responses assessed for the mildly virulent wild-type virus. Of note, a similar disparity has been found in studies on wild-type MVs of mild clinical virulence in rhesus monkeys, displaying slow kinetics of IL-4 mRNA levels in PBMCs (Devaux et al., 2008). As events in the immune induction of B-cell responses occur in lymph nodes, the local IL-4 response could give a different picture from that for PBMCs.

In the present study, we found prominent paraclinical differences between the two wild-type infections in mink. Thus, robust early neutralizing-antibody production, as assessed solely for the DK91-challenged mink, has been found to be an important correlate to recovery from CDV infections, as for MV infections (Appel et al., 1982; Forthal et al., 1992). Furthermore, transient versus irreversible lymphopenias distinguished the two wild-type infections.

The lymphocyte-associated cytokine production in our study was measured on a constant number of PBMCs and the responses appeared with late onset after the peak viraemic phase for both wild-type viruses. It should be taken into account that the severe lymphopenia provoked in the highly virulent wild-type infections is probably induced by a number of mechanisms, including virus contact-mediated inhibitions of T-cell proliferation (Griffin, 2007; Heaney et al., 2002; Yanagi et al., 2006). The effects of the latter inhibitions could, in turn, alter the ratio of cytokine-positive to-negative cells in vivo. It is also possible that different results regarding cytokine responses during acute wild-type virus infections may be obtained in further studies by use of specific antigen stimulation in vitro prior to analysis.

Moreover, for the Snyder Hill-challenged animals, all of which developed profound lymphopenia known to mirror
the extent of general lymphoid depletion in the host (Krakowka et al., 1980), the net effect of the demonstrated immune activations appeared insufficient to overcome the acute multisystemic infection.

In conclusion, our results suggest an inverse relationship between acute virulence and extent of lymphocyte-associated viraemia between the investigated wild-type CDVs in their natural host. Future studies are needed to assess to what extent reduction in the proportions of virus-infected lymphocytes undergoing rapid destruction is a key element in the attenuation of acute virulence of wild-type CDV, and the underlying mechanisms. For this purpose, studies in natural host systems, such as the one presented here in mink, appear highly relevant.

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