Acute canine distemper encephalitis is associated with rapid neuronal loss and local immune activation

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For most virus infections of the central nervous system (CNS), immune-mediated damage, the route of inoculation and death of infected cells all contribute to the pathology observed. To investigate the role of these factors in early canine distemper neuropathogenesis, we infected ferrets either intranasally or intraperitoneally with the neurovirulent canine distemper virus strain Snyder Hill. Regardless of the route of inoculation, the virus primarily targeted the olfactory bulb, brainstem, hippocampus and cerebellum, whereas only occasional foci were detected in the cortex. The infection led to widespread neuronal loss, which correlated with the clinical signs observed. Increased numbers of activated microglia, reactive gliosis and different pro-inflammatory cytokines were detected in the infected areas, suggesting that the presence and ultimate death of infected cells at early times after infection trigger strong local immune activation, despite the observed systemic immunosuppression.

INTRODUCTION

Morbilliviruses cause an acute disease characterized by generalized immunosuppression, rash, respiratory and gastrointestinal signs, and occasional but devastating neurological complications (Griffin, 2001; Moss et al., 2004; Schneider-Schaulies & Schneider-Schaulies, 2008). Within the genus Morbillivirus, carnivore viruses are associated with the highest incidence of neuroinvasion, with up to 30% of dogs and almost all wild carnivores experiencing central nervous system (CNS) infection (Appel & Summers, 1995; Confer et al., 1975; Summers et al., 1984; van Moll et al., 1995). Therefore, the study of canine distemper virus (CDV) in one of its natural hosts is frequently used to characterize mechanisms of morbillivirus neuropathogenesis (Appel et al., 1982; Headley et al., 2001; Rima et al., 1991; Summers et al., 1984).

Of the different CDV strains available, Snyder Hill causes the most reproducible course of disease including consistent neuroinvasion. In dogs infected intranasally with Snyder Hill, first clinical signs are seen after 4 days and around 50% of animals ultimately succumb to the disease (Appel, 1969; Summers et al., 1984). In those animals, virus is found in the CNS as early as 9 days after infection. At this stage, Snyder Hill displays a high affinity for cells in the grey matter throughout the brain (Appel, 1969). The resulting polioencephalitis reproduces key aspects of measles inclusion body encephalitis, which occurs in immunodeficient patients (Chadwick et al., 1982). Neurological signs, including tremors and circling behaviour, first develop after 12 days and progress rapidly to seizures or paralysis, leading to death within 2–3 weeks after infection (Appel, 1969; Summers et al., 1984). In ferrets, the virus is lethal, with all animals developing the above-mentioned neurological manifestations (Stephensen et al., 1997).

Due to the severe leukopenia and generalized immunosuppression associated with CDV infections, little inflammation has been observed in the CNS of dogs during the acute phase of disease. Local immune responses are limited to mild microglial activation and proliferation, and occasional perivascular cuffing, consisting mainly of T cells (Tipold et al., 1999; Wunschmann et al., 1999). The neurological signs and pathological changes observed are thus considered to be a direct result of the infection (Vandevelde & Zurbriggen, 2005; Vandevelde et al., 1985; Zurbriggen et al., 1998). However, the detection of different cytokine and chemokine mRNAs in the cerebrospinal fluid (CSF) of animals with chronic CDV-induced CNS lesions suggests that an aberrant local immune response may contribute importantly to disease progression (Frisk et al., 1999).

To investigate the kinetics of CDV neurodissemination and the resulting local immune response at early disease stages in more detail, we produced an enhanced green fluorescent protein (eGFP)-expressing infectious clone of the Snyder Hill strain and characterized its neuropathogenesis in ferrets. The importance of the route of inoculation and blood–brain barrier integrity were examined, and the principal target regions and cell types in the CNS at different times after infection were investigated.
infection were identified. To determine the presence and extent of the local immune response, tissue sections were stained for different markers of immune activation using newly produced antisera against pro-inflammatory ferret cytokines.

RESULTS

An eGFP-expressing derivative of the CDV Snyder Hill strain retains parental growth characteristics and virulence

To characterize the virus–host interactions involved in morbillivirus neuropathogenesis, we generated an infectious cDNA clone of the CDV Snyder Hill strain that carried the eGFP open reading frame in an additional transcription unit between the haemagglutinin (H) and polymerase (L) genes (Fig. 1a). Towards this, the parental Snyder Hill strain (ATCC VR-526) was plaque-purified on VerodogSLAMtag cells and subsequently passaged twice in ferrets, where the second ferret was inoculated with blood-free CSF collected from the first. The infectious cDNA clone represents the consensus sequence of virus isolated from the CSF of the second animal and corresponds to the sequence of the parental ATCC strain. The growth kinetics of the eGFP-expressing recombinant SHeH in VerodogSLAMtag cells were similar to the non-recombinant parental strain (Fig. 1b), and it produced a comparable cytopathic effect (data not shown).

To determine whether SHeH had retained the overall virulence and neurotropism of the parental strain, a group of four ferrets was infected intranasally with $10^5$ 50% tissue culture infectious doses ($TCID_{50}$) of either SH or SHeH. Onset and severity of clinical signs were similar in both groups (Fig. 1c) and all animals developed neurological signs. At least half of the animals displayed nausea and vomiting, suggestive of meningitis, starting as early as 6 days after infection. Facial spasms or disordered movement indicative of cerebellar involvement was observed in the majority of animals at later disease stages. All SH- or SHeH-infected animals succumbed to the disease within 12–16 days after infection, thereby reproducing the clinical course and disease duration seen in animals infected with the original ATCC virus (data not shown). Macroscopic examination of the brains at the time of death revealed strong infection of the olfactory bulb (Fig. 1d), and widespread eGFP expression in olfactory nerves and mitral cells was observed microscopically (Fig. 1e). Taken together, these analyses indicate that the recombinant eGFP-expressing virus reproduces the parental disease phenotype.

CDV neuroinvasion is independent of the inoculation route and does not require blood–brain barrier damage

Using different eGFP-expressing CDV strains, we have shown previously that the virus first targets lymphatic tissues and organs and then spreads to epithelia and the CNS (Rudd et al., 2006; von Messling et al., 2004). However, the anatomical proximity of the olfactory bulb to...
the nasal cavities raises the possibility that intranasal inoculation may lead to direct infection of olfactory neurons, thereby facilitating neuroinvasion. To investigate the importance of the route of inoculation for CDV neuroinvasion, we compared disease progression and dissemination in animals infected intraperitoneally or intranasally with $10^5$ TCID$_{50}$ SHeH. All animals showed first signs of rash and fever around 6 days after infection and had to be euthanized around day 14 (Fig. 2a). Starting at 7 days after infection, severe leukopenia and inhibition of PBMC proliferation activity following phytohaemagglutinin (PHA) stimulation were observed in both groups (Fig. 2b, c), and the kinetics of the cell-associated viraemia were similar (Fig. 2d). No differences were noted with respect to overall virus distribution in the brain (data not shown), and eGFP expression in the olfactory bulbs was first detected macroscopically at day 14 in the final disease stage (Fig. 2e), regardless of the route of inoculation. Since it had also been suggested that infection-induced damage of the blood–brain barrier contributes to neuroinvasion (Axthelm & Krakowka, 1987), we assessed blood–brain barrier integrity over the course of the infection.

Towards this, animals received an intracardiac injection of Evans blue, which is unable to cross an intact blood–brain barrier due to its large molecular mass, 45 min before sacrifice at different times after infection. Diffuse staining of the brain was only observed at the day 14 time point (Fig. 3), when CNS infection was already macroscopically detectable (Figs 1d and 2e) and the animals were showing neurological signs, indicating that CDV neuroinvasion occurs without damaging the blood–brain barrier severely.

**Fig. 2.** Comparison of intranasal (IN) and intraperitoneal (IP) inoculation. (a) Survival curve of groups of four ferrets infected with $10^5$ TCID$_{50}$ SHeH either intranasally (○) or intraperitoneally (■). Animals that reached experimental end points were euthanized. The time of death is represented by a step down in the graph. (b–d) Leukocyte numbers (b), in vitro proliferation activity (c) and cell-associated viraemia (d) are depicted for both groups of animals. Time post-infection is indicated on the x-axis and leukocyte numbers, proliferation activity or number of CDV-infected cells per million PBMCs are indicated on the y-axis. (e) Visualization of olfactory bulb infection. Macroscopic eGFP expression is first detected 14 days after infection regardless of the route of inoculation. Olfactory bulb contours are outlined by a white line.

**SHeH infection results in substantial neuronal damage and gliosis**

To assess the kinetics of neuroinvasion and identify target areas, two or three animals were sacrificed at days 7, 10 and 14 after infection and sagittal brain sections were stained for the presence of CDV-infected cells. The first infected cells were detected in the cerebellum and the olfactory bulb after 7 days, and a continuous increase was observed as the disease progressed (Fig. 4a). Similar infection kinetics were also observed in the choroid plexus, hippocampus and brainstem, whereas only occasional foci were seen in the...
cerebrum (data not shown). Within these regions, neurons in general, but particularly Purkinje and granular cells, were targeted preferentially. Evaluation of the onset and extent of histopathological changes revealed little infiltration of mononuclear cells, indicative of an inflammatory response over the course of the infection. Even at the time of death, only occasional foci were found, mostly in close proximity to highly infected areas (Fig. 4b). Instead, many infected neurons underwent morphological changes indicative of severe neuronal injury, including acute neuronal necrosis (Fig. 4c), atrophy (data not shown) and ballooning resembling karyolysis (Fig. 4d). Neuronophagia was also occasionally observed (data not shown).

To determine whether these infection-induced morphological changes ultimately resulted in cell death, we focused on the cerebellum, where the first infected Purkinje cells were found on day 7 (Fig. 4a, top row). A progressive increase of infection and loss of Purkinje cells were observed, resulting in a 35% reduction and a threefold increase in distance between neighbouring cells at the time of death (Fig. 4a, top row; Fig. 5a, b). TUNEL staining revealed no increase in apoptosis in the different brain areas over the course of the infection (Fig. 5c), suggesting that virus-induced cell death occurs via an alternative mechanism.

**Pro-inflammatory cytokines are present in the CNS**

CDV causes severe leukopenia and T and B lymphocytes are one of its main targets, with infection levels over 70% within the first week (von Messling et al., 2004). It is thus not surprising that few signs of an inflammatory response, such as lymphocyte infiltration and perivascular cuffing, are seen. To investigate whether the infection results in local immune activation, we evaluated the extent of gliosis, an indicator of neuroinflammation, and the presence of activated microglial cells and of cells expressing beta interferon (IFN-β) and pro-inflammatory cytokines. Reactive gliosis, as indicated by an increase in glial fibrillary acidic protein (GFAP)-positive cells, was detected in areas surrounding infected cells within the first week and continued as the infection progressed (Fig. 6a). A limited number of activated microglia were found at early infection stages, but their presence diminished as the infection progressed (Fig. 6b). Similar kinetics were observed for interleukin-6 (IL-6), whilst a gradual increase of IFN-β- and tumour necrosis factor alpha (TNF-α)-expressing cells was seen (Fig. 7), many of which were infected (Fig. 8; data not shown). This local TNF-α expression may contribute to the breakdown of the blood–brain barrier observed at late infection stages (Fig. 3).

**DISCUSSION**

In addition to the CNS diseases caused by primarily neurotropic viruses, neurological complications are associated with a number of viral infections (Acharya & Pacheco, 2008; Gnann, 2002; Letendre et al., 2009; Whitley & Gnann, 2002). Once these viruses succeed in invading the CNS, damage caused by the death of infected cells is often amplified by the local immune response. This immune activation may persist long after the viral infection has been resolved and may lead to continued neuronal injury and loss (Stoica et al., 2000) or contribute to neurodegeneration by affecting other CNS cell populations. As some morbillivirus neurological complications occur in the context of genetic or acquired immunodeficiencies, whilst others are associated with an excessive inflammatory response, we investigated the contribution of infection and immune response to CDV neuropathogenesis during the early infection phase. We show that the virus targets cells in theolfactory bulb, brainstem, hippocampus and cerebellum, regardless of the route of inoculation. Development of neurological signs, especially motor deficits, correlates with the progressive loss of infected neurons in these areas. Only a mild transitory inflammatory response is detected at early stages, despite the presence of pro-inflammatory cytokines.
Even though insufficient in the context of lethal systemic disease, this local cytokine expression, in combination with inflammatory mediators released upon non-apoptotic death of the infected neurons, may trigger the aberrant inflammatory response observed in chronic neurological complications associated with morbillivirus infections.

**Inoculation route does not influence morbillivirus dissemination**

For many neurotropic viruses, the point of entry in the periphery determines the time course of neuroinvasion and even the CNS area affected (Anderson & Field, 1983; Kuss...
et al., 2008; Sinchaisri et al., 1992). Previous studies have shown that, upon intranasal inoculation, immune cells are the initial morbillivirus targets, followed by spread to epithelia and the CNS (de Swart et al., 2007; von Messling et al., 2004). In addition to the well-characterized hematogenous route (Higgins et al., 1982), we demonstrated that the olfactory bulb is a main point of morbillivirus CNS entry (Rudd et al., 2006). The similar CDV neuroinvasion kinetics observed upon intranasal and intraperitoneal inoculation indicate that this sequential dissemination constitutes an integral part of morbillivirus pathogenesis. The most likely explanation for this phenomenon is that immune cells expressing the morbillivirus high-affinity receptor, the signalling lymphocyte activation molecule (SLAM, CD150) (Cocks et al., 1995; Sidorenko & Clark, 2003; Tatsuo et al., 2001), are equally available in the upper respiratory tract and the peritoneal cavity. The importance of this initial infection of immune cells has been illustrated by the complete attenuation of SLAM-blind viruses in vivo (von Messling et al., 2006). Once the infection is established in the immune system, subsequent spread to epithelia and the CNS is independent of the original site of inoculation.

**Morbilliviruses are unable to prevent local immune activation in the CNS**

The immunosuppressive effects of morbilliviruses on immune cells have been studied extensively in vitro and in vivo. Contact with the viral glycoproteins inhibits lymphocyte proliferation (Sanchez-Lanier et al., 1988; Schlender et al., 1996), and the V protein efficiently prevents innate immune activation and induction of an antiviral state (Caignard et al., 2007; Ohno et al., 2004; Palosaari et al., 2003; Takeuchi et al., 2003). Lethal disease is characterized by severe leukopenia and a complete lack of cytokine induction and loss of proliferation capacity in PBMCs (Svitk & von Messling, 2007). Here, we observed an immune activation in resident CNS cells that increased as the infection progressed, suggesting that the virus is unable
to block an innate tissue response. A similar tissue response in epithelia may explain the apparent dichotomy of an efficient cellular and humoral antiviral response in the presence of generalized immunosuppression observed in uncomplicated measles virus (MeV) and other non-fatal morbillivirus infections.

Distemper encephalomyelitis – an unwanted consequence of early CNS immune activation?

In the context of viral infections, pro-inflammatory cytokines and chemokines released upon non-physiological death of infected cells usually result in recruitment of circulating immune cells that ultimately clear the infection. However, due to the severe leukopenia associated with morbillivirus infections, only very few immune cells are available for recruitment to the sites of injury during the acute disease phase, explaining the general lack of infiltration. Time-course studies and retrospective analyses of natural cases in dogs indicate that CDV demyelinating leukoencephalomyelitis, which occurs weeks to years after recovery from the acute disease, involves a two-step process: the initial damage during the acute disease phase is caused directly by the virus, whereas plaque progression after the recovery of the immune system is thought to be primarily immune-mediated (Allinger et al., 1996; Baumgartner et al., 1989; Summers & Appel, 1994). Our observation of widespread death of infected neurons coinciding with sustained local immune activation in the context of a fatal disease supports this model, but suggests that the aberrant inflammatory response seen after virus clearance may be triggered by this early immune activation.

METHODS

Cells and viruses. VerodogSLAMtag cells and 293 cells (ATCC CRL-1573) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with 5% fetal bovine serum (FBS; Invitrogen). The CDV Snyder Hill strain (ATCC VR-326) and all recombinant

Fig. 7. Presence of pro-inflammatory cytokines in the cerebellum. Beta interferon (IFN-β, left column), interleukin-6 (IL-6, middle column), and tumour necrosis factor alpha (TNF-α, right column) were detected in paraffin sections of the cerebellum using polyclonal antibodies raised in chicken directed against the respective ferret cytokine. All slides were counterstained with haematoxylin. Stars highlight positive cells. Original magnification, ×400; bar, 50 μm.

Fig. 8. Co-localization of CDV and IFN-β in the cerebellum. Paraffin sections were incubated with an antiserum directed against IFN-β raised in rabbits and visualized by using an Alexa Fluor 568-labelled secondary antibody (red). Infected cells were stained with a monoclonal antibody recognizing CDV N followed by an Alexa Fluor 488-labelled secondary antibody (green). Merge images are shown of representative sections from a non-infected control animal (upper left) and of animals sacrificed on days 7 (upper right), 10 (lower left) and 14 (lower right) post-infection. Original magnification, ×400 (insets, ×1000). Nuclei were stained using DAPI. Bars, 20 μm.
viruses produced were propagated in VerodogSLAMtag cells. For plaque purification, the ATCC inoculum was diluted serially in tenfold steps and added to VerodogSLAMtag cells seeded in six-well plates. After 30 min at 37 °C, medium was replaced with a 2 % agar overlay and the plates were incubated for 3 days. Individual syncytia were picked and virus stocks were produced.

Construction and recovery of recombinant viruses. To generate an infectious cDNA clone of the CDV Snyder Hill strain, RNA was isolated from VerodogSLAMtag cells infected with CSF obtained from the last infected ferret (see detailed description in the following section) using an RNeasy mini kit (Qiagen). The cloning strategy used followed that described previously for other CDV strains (von Messling et al., 2003). Briefly, the RNA was reverse-transcribed using Superscript II (Invitrogen) and the complete genome was amplified in ten separate fragments using high-fidelity polymerase (Roche) and subcloned into PCR2.1-TOPO (Invitrogen). At least four clones of each fragment were sequenced to establish the consensus sequence. The viral genomic cDNA clone was assembled from fragments corresponding to the consensus sequence by using naturally occurring unique restriction sites, yielding pSH (GenBank accession no. GU138403). The eGFP open reading frame was then introduced as an additional transcription unit between the H and L genes following the same approach as for 5804PeH (von Messling et al., 2004), yielding pSHEH.

Recombinant viruses were recovered by using a vaccinia-free system (Anderson & von Messling, 2008; Martin et al., 2006). Towards this, semi-confluent 293 cells seeded in six-well plates were transfected with 4 μg plasmid carrying the full-length genome, 0.5 μg MeV nucleoprotein (N), 0.1 μg MeV phosphoprotein (P), 0.5 μg MeV L and 0.7 μg T7 polymerase expression plasmids by using Lipofectamine 2000 (Invitrogen). Two days post-transfection, 293 cells were overlaid onto 10 cm culture dishes containing a confluent monolayer of VerodogSLAMtag cells. The resulting co-cultures were maintained in DMEM containing 5 % FBS until syncytia were observed. Virus stocks were then produced by transferring individual syncytia onto fresh VerodogSLAMtag cells. Multi-step growth curves were performed by infecting VerodogSLAMtag cells with an m.o.i. of 0.01 of the respective strain and incubating them at 32 °C. Cells and supernatant were harvested for 5 days; virus titres were determined by infecting VerodogSLAMtag cells with an m.o.i. of 0.01 of the respective strain and incubating them at 32 °C. Cell-associated viraemia was quantified in PBMCs isolated by lysing erythrocytes in EDTA-treated blood in ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.01 mM EDTA, pH 7.2–7.4) and co-cultivated with VerodogSLAMtag cells in quadruplicates of 10-fold serial dilutions. Wells were evaluated for cytopathic effect after 3 days, and titres were expressed as 50 % TCID50 per 106 cells.

Assessment of immunosuppressive activity. For the total white blood cell count, 10 μl heparinized blood was added to 990 μl of a 3 % solution of acetic acid, and nucleated cells were counted. The proliferation activity was determined by using the 3-bromo-2'-deoxyuridine (BrDU) cell proliferation assay (Roche) according to the manufacturer’s instructions. Briefly, the PBMCs from each animal, isolated by Ficoll (GE Healthcare) gradient centrifugation, were split into two duplicates and either stimulated with 2 μg PHA ml−1 (Sigma) or left untreated. After 24 h incubation, BrDU was added to a final concentration of 10 μM, and cells were incubated for another 24 h. Cells were then transferred into a black 96-well plate, washed and fixed at 65 °C for 1 h. BrDU incorporation was detected by using a peroxidase-coupled anti-BrDU antibody and a chemiluminescent substrate. The proliferation activity was expressed as a ratio between stimulated and non-stimulated cells, allowing for comparison of samples that differ in absolute cell numbers due to the virus-induced leukenopaenia. Cell-associated viraemia was quantified in PBMCs isolated by lysing erythrocytes in EDTA-treated blood in ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.01 mM EDTA, pH 7.2–7.4) and co-cultivated with VerodogSLAMtag cells in quadruplicates of 10-fold serial dilutions. Wells were evaluated for cytopathic effect after 3 days, and titres were expressed as 50 % TCID50 per 106 cells.

Assessment of blood–brain barrier integrity. Animals were anaesthetized and injected intracardially with 5 ml of a 2 % (w/v) Evans blue (Sigma) solution diluted in 0.9 % NaCl. Forty-five minutes later, animals were euthanized and perfused with PBS (Invitrogen) until the drainage was colourless. For the positive control, the animal was first injected intracardially with 10 ml of a 20 % mannitol solution, followed by the Evans blue injection. Ventral and dorsal views of the brains were imaged under normal light by using the Macro-illumination imaging system (Lichtools).

Preparation of antigens and production of polyclonal antibodies. To produce purified proteins, ferret cytokines were amplified as described previously (Svitik & von Messling, 2007). PCR products were cloned into the pET32a vector (Novagen), confirmed by sequencing and transformed into Escherichia coli BL21 (DE3) competent cells (Novagen) for expression. Proteins were then purified by electrophoresis and extracted by using 0.1 % SDS. Antigenic sequences were chosen by the Jameson–Wolf index, yielding the following peptides: IFN-β, CLKDRMNFKIEEIKDSQ; IL-6, CGDSKDDATSNRPLTSA; TNF-α, CVKSSHRTPSDKPV. Rabbit and chicken antisera were raised against either purified bacterial proteins or synthetic peptides, respectively, following the standard protocol (Cocalico Biologicals).

Cryosections and immunohistochemistry. Animals were euthanized with an overdose of intraperitoneal pentobarbital (CDMV, Inc.). Each animal was perfused first with 160 ml PBS (Invitrogen), followed by 80 ml 4 % paraformaldehyde (PFA; Electron Microscopy Sciences). Tissues were harvested, fixed in 4 % PFA for at least 24 h at 4 °C and stored in PBS. Prior to sectioning, samples were placed in 30 % sucrose in PBS overnight at 4 °C, immersed in tissue embedding compound (Triangle Biomedical Sciences) and frozen on dry ice for at least 1 h. Serial 5–10 μm sections were cut by using a cryostat (Kryostat 1720 digital; Leitz) and mounted on Superfrost Plus slides (Fisher Scientific), air-dried and stored at 20 °C. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and coverslips were

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mounted with Prolong Gold antifade reagent (Invitrogen) and left to harden overnight at 4°C.

For immunohistochemistry staining, paraffin sections were deparaffinized and rehydrated following standard immunohistochemistry protocols. Endogenous peroxidase was quenched with 0.3% H2O2 in PBS for 12 min. For GFAP staining, the slides were incubated in 10 mM sodium citrate (pH 6) solution (Fisher Scientific) and microwaved for 15 min before being transferred into PBS. After blocking with a 1:50 dilution of horse serum in PBS, the respective primary antibody [rabbit-anti GFAP (DAKO), mouse ascitic fluid against rat Mac-2 (kind gift from Dr Pierre Talbot, INRS-Institut Armand-Frappier), mouse monoclonal antibody against the CDV N protein (VMRD) or chicken hyperimmune serum against the respective ferret cytokine] was added for 1 h at room temperature, followed by incubation with an appropriate biotinylated secondary antibody and subsequently peroxidase-labelled streptavidin (Vector Laboratories), each for 45 min at room temperature. Positive cells were visualized by using 3,3'-diaminobenzidine (DAB) substrate (Sigma) and slides were counterstained in haematoxylin. For haematoxylin/eosin staining, paraffin-embedded sections were incubated for 30 min in xylene and then rehydrated prior to staining for 5 min in Harris’ haematoxylin solution (EMD Industries). Sections were rinsed in double-distilled H2O, dipped in a 0.3% ammonia solution and counterstained with acidified eosin Y (Sigma). Slides were dehydrated, mounted in Entellan mounting medium (EMD Industries) and air-dried overnight.

For double staining, slides were first incubated with the rabbit hyperimmune serum against the respective cytokine, followed by an Alexa Fluor 568-labelled secondary antibody (Invitrogen), and then permeabilized using 0.1% sodium citrate and 0.1% Triton X-100 dissolved in water for 8 min at room temperature, rinsed twice in PBS and incubated with the TUNEL reaction mixture for 1 h at 37°C. Slides were washed twice with PBS and counterstained with DAPI. The positive control was obtained by treating slides with 0.5 U DNase I (Fermentas) for 10 min at room temperature before adding the TUNEL reaction mixture. All slides were analysed by fluorescence microscopy.

**TUNEL assay.** The extent of apoptosis in the tissue was determined by TUNEL assay, using an In situ cell death detection kit, POD (Roche) according to the manufacturer’s instructions. Briefly, 5 μm thick paraffin-embedded sections were deparaffinized and rehydrated following standard immunohistochemistry protocols. Slides were then permeabilized using 0.1% sodium citrate and 0.1% Triton X-100 dissolved in water for 8 min at room temperature, rinsed twice in PBS and incubated with the TUNEL reaction mixture for 1 h at 37°C. Slides were washed twice with PBS and counterstained with DAPI. The positive control was obtained by treating slides with 0.5 U DNase I (Fermentas) for 10 min at room temperature before adding the TUNEL reaction mixture. All slides were analysed by fluorescence microscopy.

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