Canine Distemper Virus Selectively Inhibits Apoptosis Progression in Infected Immune Cells

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Morbillivirus infections are characterized by severe leukopenia and immune suppression that develop even before the onset of clinical signs. To characterize in more detail the fate of the immune cells during the critical first week, we evaluated the overall viability, level of apoptosis, cell cycle status, and extent of infection in different immune tissues of ferrets inoculated with a lethal canine distemper virus (CDV) strain. Initial experiments with MDCK cells, a canine epithelial cell line, revealed that CDV infection resulted in only a marginal increase in apoptosis at high infection levels and that infected cells were more resistant to chemically induced apoptosis. In ferrets, levels of viability and early and late apoptosis remained stable in thymus and lymph node, where more than 80% of cells were infected, whereas a gradual albeit small increase in apoptosis was observed in peripheral blood mononuclear cells and spleen. Furthermore, the progression of spontaneous apoptosis in infected cells was inhibited, while the proportion of apoptotic noninfected “bystander” cells increased. The distribution of cells in the different stages of the cell cycle in the bone marrow was not affected, but dividing cells in the thymus decreased by 50%, and a 10-fold increase in cell division was noted in the spleen. It is unlikely that the extent of infection-induced cell death and cell cycle alterations alone can account for the dramatic leukopenia observed in this model. The investigation of additional mechanisms is therefore warranted.

Morbilliviruses are highly contagious pathogens that cause systemic disease. In addition to respiratory and gastrointestinal signs, the disease is characterized by a rapid onset of severe leukopenia and loss of lymphocyte proliferation ability. The resulting immunosuppression increases the host’s susceptibility to opportunistic infections, which are a main cause of morbillivirus-associated deaths (4, 15, 29). In the case of measles virus (MeV), which infects only humans and certain nonhuman primates, fatal disease outcome is rare (14), while some animal morbilliviruses, especially those infecting carnivores, can approach 100% mortality (3). The characterization of the events leading to immunosuppression has been the subject of intense study. The signaling lymphocyte activation molecule (SLAM, CD150), the only general morbillivirus receptor identified so far, is present on lymphocyte subsets, which explains the lymphotropism observed (36, 40). However, the effects of the infection on infected as well as noninfected immune cells during the critical first week after infection, before the development of clinical signs, remain largely unknown.

Virus-induced apoptosis of immune cells has been proposed as one of the causes of the severe leukopenia observed (25). Apoptosis is a physiological process of cell death that is essential for normal tissue turnover during embryogenesis, immune system development, and tissue homeostasis. It also constitutes a basic antiviral mechanism that limits replication and spread by driving suicide of infected cells (1, 6). MeV infection results in apoptosis in Vero cells and human monocytic or promonocytic cell lines (10), as well as in primary cultured monocytes and dendritic cells (12). This capacity to induce apoptosis in infected cells and the resulting presentation of tumor antigens is increasingly exploited in oncolytic gene therapy approaches (13, 21).

In addition, bystander apoptosis of noninfected CD3+ cells has been observed after in vitro infection of human peripheral blood mononuclear cells (PBMCs) (12, 41). These observations have been supported by direct analyses of PBMCs from MeV patients, where high levels of proapoptotic noninfected cells were found (25, 26). In the cotton rat model, exposure to MeV glycoproteins initially resulted in cell cycle arrest in splenocytes (23), indicating that contact with viral proteins may be the initial trigger of bystander apoptosis. Increased apoptosis has also been observed in lymphatic tissue sections from cattle experimentally infected with rinderpest virus and in brain sections of dogs with nervous distemper (5, 33). However, the infection status of these cells remains to be determined.

Most viruses have developed mechanisms to prevent or at least control apoptosis (6), since apoptotic cells are rapidly phagocytosed, which leads to the presentation of viral antigens, thereby supporting the development of an efficient immune response (1, 11, 27). The MeV V protein exhibits antiapoptotic ability by inhibiting p73, a member of the p53 family that is strongly involved in the regulation of apoptosis (7), and it has recently been reported that the C protein interferes with apoptosis induction by blocking the protein kinase regulated by RNA (PKR) (37). It is thus likely that the disease course observed is the result of pro- and antiapoptotic events occurring simultaneously.

To characterize the contribution of apoptosis to morbillivirus immunopathogenesis in more detail, we studied an enhanced green fluorescent protein (eGFP)-expressing wild-type canine distemper virus (CDV) strain in ferrets. This strain reliably causes severe leukopenia, inhibition of lymphocyte

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proliferation, and loss of delayed-type hypersensitivity responses (39), thus reproducing key elements of MeV immunosuppression. We initially examined the effect of CDV infection in Madin-Darby canine kidney (MDCK) cells, a canine epithelial cell line, to assess the extent of apoptosis in a natural target cell type in vitro. This was followed by a time course analysis of immune tissues covering the first week after infection. Specifically, infection rates, cell viability, cell cycle distribution, and apoptosis were assessed in bone marrow, thymus, spleen, mesenteric lymph node, and PBMCs of infected ferrets.

MATERIALS AND METHODS

Cells, viruses, and in vitro infections. MDCK cells (ATCC, CCL-34) and VerodosoSLAMtag cells (39) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 5% heat-inactivated fetal calf serum (Invitrogen). The CDV wild-type strain 5804PeH (38) was grown in VerodosoSLAMtag cells. It carries an additional transcription unit coding for the gFP in its genome, allowing for the direct identification of infected cells. For all in vitro studies, MDCK cells were seeded in either six-well plates or chamber slides (Nalgene) and infected with virus at a multiplicity of infection (MOI) of 1. To obtain high infection levels, infected cells were passaged twice weekly for up to 4 weeks. Where indicated, 1 μM of staurosporine (Sigma) was added during the last 4 h of incubation.

Apoptosis assays and cell cycle analysis. The in situ cell death detection kit, TMR red (Roche), was used to detect DNA fragmentation according to the manufacturer’s instructions. Briefly, cells suspensions or adherent cells were fixed with phosphate-buffered saline (PBS) (Invitrogen) containing 2% and 4% paraformaldehyde (Sigma), respectively. Cells were washed with PBS and permeabilized with a solution of 0.1% Triton X-100 in 0.1% sodium citrate. DNA strand breaks were labeled with terminal deoxynucleotidyl transferase (TUNEL) (Roche) marked with a red fluorescent dye. Stained cells were then analyzed by flow cytometry (FACSCalibur; BD Biosciences) or examined under a fluorescence microscope (Nikon Eclipse 80i). Photographs were taken with a digital camera (Nikon DXM1200F).

Phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane was detected using a phycoerythrin-conjugated annexin V (AnV) detection kit (BD Pharmingen) following the manufacturer’s instructions. The combination of AnV-phycoerythrin and 7-amino-actinomycin D (7-AAD) staining allowed the discrimination of viable (AnV-negative, 7-AAD-negative), early apoptotic (AnV-positive, 7-AAD-negative), and dead (AnV-positive, 7-AAD-positive) cells by flow cytometry.

For the cell cycle analysis, cells were fixed in PBS containing 1% paraformaldehyde for 1 h at 4°C. After washes with cold PBS, cells were resuspended in 500 μl of ice-cold 70% ethanol under agitation and stored overnight at 4°C. Cells were then washed twice and fixed for 1 h in 70% ethanol 40 μg/ml of propidium iodide (Invitrogen) and 100 μg/ml of DNase-free RNase A (Fermentas) in PBS. The cell cycle distribution was determined by flow cytometry.

Polyacrylamide gel electrophoresis and Western blotting. Cells were washed with cold PBS and lysed on ice with 200 μl of radioimmunoprecipitation assay buffer (1 mM phenylmethylsulfonyl fluoride, 1% sodium deoxycholate, 50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl) supplemented with protease inhibitors following the manufacturer’s instructions (Complete Mini; Roche). The cell lysate was clarified by centrifugation at 17,000 × g for 10 min at 4°C, and the supernatant was mixed with 1 volume of loading buffer, incubated for 10 min at 65°C, and loaded on a 12% polyacrylamide gel. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and semidyry transfer, Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation) were blocked overnight in blocking buffer (Roche) and sequentially incubated with rabbit hyperimmune sera against a synthetic peptide corresponding to the capsae 3 cleavage site (CS487; Sigma), and actin (A2066; Sigma), followed by a horseradish peroxidase-conjugated secondary antibody (Sigma). Proteins were visualized using the ECL Plus Western blotting detection system (GE Healthcare) and exposed on a luminescent image analyzer (Kodak).

Differential peripheral blood leukocytes and lymphocyte subpopulation count. An aliquot of heparinized blood was diluted 1:100 in a fixative solution of 1% paraformaldehyde in PBS and stored at 4°C for up to 2 weeks. One volume of fixed blood was mixed with the same volume of 10 μM DRAQ5 in PBS (Biotostatus), flow cytometry absolute count standard beads (Bangs Laboratories) were added to the solution to reach a final dilution of beads of 1/10, and the sample was immediately analyzed by flow cytometry. Nucleated cells were identified by DRAQ5 staining, and the different white blood cell populations were gated based on their location in size/granularity scatter plots. At least 5,000 beads were acquired. The concentration of beads certified by manufacturer allowed the calculation of the exact volume corresponding to the number of cells acquired.

For the analysis of lymphocyte populations, PBMCs were stained with phycoerythrin-conjugated monoclonal antibodies against CD3 (sc-20047; Santa Cruz Biotechnology), CD79a (sc-20064; Santa Cruz Biotechnology), or CD14 (CAM36A; VMRD), which cross-react with ferret immune T cells, B cells, and monocytes, respectively (38).

Animal experiments. Unvaccinated adult male ferrets (Mustela putorius furo; Marshall Farms) were used for the study. The animal experiments were carried out as described previously (39) and were approved by the Institutional Animal Care and Use Committee of the INRS, Institut Armand-Frappier. Groups of three or four animals were infected intranasally with 10^5 50% tissue culture infective doses of 5804PeH. Body temperature and clinical signs were recorded daily. Animal were euthanized at 0, 3, 5, or 7 days after infection, and bone marrow, thymus, spleen, mesenteric lymph node, and peripheral blood were harvested. PBMCs were isolated by Ficoll (GE Healthcare) gradient centrifugation as previously described (38). Cell suspensions from thymus, spleen, bone marrow, and mesenteric lymph node were obtained by passing the tissue through a nylon cell strainer with a 100-μm pore size (BD Biosciences) in Leibovitz’s medium (L-15; Invitrogen), followed by Ficoll gradient centrifugation at 400 × g for 40 min. All cells were ultimately resuspended in RPMI (Invitrogen) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin (Invitrogen), and 100 IU penicillin (Invitrogen). Viable cells were counted using ethidium bromide and acridine orange dye uptake staining, and cell suspensions were adjusted at 10^8 viable cells/ml. AnV and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed on 2.5 × 10^6 and 10^7 cells, respectively. In addition, 10^6 cells were seeded in duplicate in 48-well plates and incubated at 37°C for 18 h.

Statistical methods. The statistical analyses were performed using GraphPad Prism 5.01. Effects of the infection during the first week were assessed by a one-way analysis of variance followed by Tukey’s posteriori test to determine significant differences between the time points. The significance of the differences between infected and noninfected cells was determined by the unpaired Student t test.

RESULTS

CDV infection marginally increases apoptosis in canine epithelial cells. It has been previously reported that CDV induces apoptosis in Vero cells through caspase 3 activation, which was largely limited to infected cells (18). Since antiapoptotic mechanisms may be host specific, we investigated the effect of CDV infection on apoptosis in MDCK cells, a canine kidney cell line. Cells were infected at an MOI of 1 with the wild-type strain 5804PeH, and TUNEL assays were performed after 4 days, when 10 to 15% infection levels were reached (Fig. 1A, middle panels). To assess the incidence of apoptosis at higher infection levels, cells were passaged serially and TUNEL stained when 75% of cells were gFP positive (Fig. 1A, bottom panels). Only rare individual TUNEL-positive cells were detected in both infected and mock-infected cell cultures, and infected cells were generally not apoptotic (Fig. 1A). A slight increase in caspase 3 activation was observed at high infection levels (Fig. 1B), and cytometric analysis revealed a small augmentation from 1 to 4% in overall apoptosis in highly infected cultures (Fig. 1C, middle column).

CDV reduces chemically induced apoptosis. To determine if CDV, similar to other paramyxoviruses (35, 34), has the ability to block apoptosis, infected cultures were treated with 1 μM staurosporine, which induces apoptosis via inhibition of protein kinase C and interference with the cell cycle machinery (17). Treatment of mock-infected cultures resulted in 50% or more TUNEL-positive cells and strong caspase 3 activation,
demonstrating that the apoptotic pathways in MDCK cells are functional (Fig. 1B and C). Similar values were observed at low infection levels, while an increase of infection to 75% correlated with the reduction of apoptosis upon staurosporine treatment to around 20%.

Systematic evaluation under high-power magnification showed no preferential colocalization of infected and apoptotic cells in mock- or staurosporine-treated cultures (Fig. 2). Quantification of infected and noninfected apoptotic cells by flow cytometry revealed that in the presence of staurosporine (stauro) were used as a positive control. (C) Quantification of infection and apoptosis levels. MDCK cultures at different infection levels were incubated for 4 h with 1 μM staurosporine, detached, and TUNEL stained. The proportions of eGFP-positive and TUNEL-positive MDCK cells (mean ± standard error) were assessed by flow cytometry. The data shown are representative of at least three independent experiments.

FIG. 1. Apoptosis assessment in CDV-infected MDCK cells. MDCK cells, a canine kidney epithelial cell line, were infected with 5804PeH at an MOI of 1, as per titration on VerodogSLAMtag cells. (A) Immunofluorescence staining for DNA fragmentation by TUNEL assay. Mock- and 5804PeH-infected cells were fixed either at 4 days postinfection (upper and middle panels) or after consecutive passages when a 75% infection rate was reached (lower panels) and TUNEL stained (right column). Infected cells were identified by eGFP expression (middle column), and all cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (left column). Photographs were taken at a magnification of ×400. (B) Western blot analysis of activated caspase 3. Infected MDCK cells were harvested at 4 days postinfection, corresponding to an infection rate of 15% (+), or after consecutive passages when 75% infection was reached (+++). Cell lysates were analyzed for the presence of activated caspase 3 using a rabbit hyperimmune serum specific for a synthetic peptide corresponding to the cleavage site of caspase 3 and with rabbit anti-actin antibody, followed by an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody. MDCK cells incubated 4 h with 1 μM staurosporine (stauro) were used as a positive control. (C) Quantification of infection and apoptosis levels. MDCK cultures at different infection levels were incubated for 4 h with 1 μM staurosporine, detached, and TUNEL stained. The proportions of eGFP-positive and TUNEL-positive MDCK cells (mean ± standard error) were assessed by flow cytometry. The data shown are representative of at least three independent experiments.

Infected immune cells remain viable despite severe leukopenia. In ferrets, the infection level in immune cells increases dramatically during the first week of CDV infection, coinciding with the development of a severe leukopenia (38), and a similar if less extreme kinetic has been observed in primates experimentally infected with MeV (2, 8). To investigate the impact of CDV infection on cell viability, unvaccinated male ferrets were infected intranasally with 10^5 50% tissue culture infective doses of 5804PeH. The proportions of eGFP-positive cells in bone marrow, thymus, spleen, mesenteric lymph node, and PBMCs were determined by flow cytometry at 0, 3, 5, and 7 days postinfection (Fig. 3A, left panels). Consistent with previous findings, infected cells were first detected after 3 days.
With the exception of the lymph node, little change in infection levels was observed between days 3 and 5 after infection. After 7 days, more than 80% of cells residing in the thymus and lymph node were infected, while around 50% of PBMCs and fewer than 20% of bone marrow and spleen cells were eGFP positive (Fig. 3A, left panels). No significant changes in cell viability, based on 7-AAD staining, were observed in bone marrow, thymus, spleen, or PBMCs (Fig. 3A, middle panels). The proportion of live cells in the lymph node, where around 70% viability was seen in noninfected animals, increased concomitantly with the infection levels (Fig. 3A, middle panels). Analysis by infection status revealed that the overwhelming majority of infected cells were alive, regardless of the tissue (Fig. 3A, right panels).

To accurately assess the extent of infection-induced leukopenia during the same time period, a no-lyse, no-wash flow cytometry protocol was developed. A major drop in the number of granulocytes from 5,000 cells/l to 1,000 cells/l was observed within the first 3 days of infection. This was followed by the reduction of PBMCs from 4,000 cells/l to 1,000 cells/l between days 3 and 5 after infection, when the proportion of infected cells was still low (Fig. 3A and B, left panels). The leukopenia progressed further from day 5 to day 7, leaving overall white blood cell numbers at 2,500 cells/µl and PBMC counts at fewer than 500 cells/µl, corresponding to around 25% and 10% of preinfection levels, respectively. Since the most dramatic loss was seen in PBMCs, the effects on the different subpopulations were evaluated. T cells declined within the first week of infection from 3,000 cells/µl in noninfected animals to below 250 cells/µl, corresponding to a loss of 90% of the population, with infection levels reaching more than 50%. The total number of B cells dropped from 1,700 cells/µl to around 300 cells/µl, with up to 75% infection, while monocytes declined by 30% from 350 to 100 cells/µl even though infection levels never exceeded 15% (Fig. 3B, right panel). Thus, CDV infection results in a substantial loss of all leukocyte populations, regardless of their extent of infection.

The impact of infection on the cell cycle status is tissue dependent. As cell cycle arrest after contact with viral glycoproteins has been demonstrated for MeV in vitro and ex vivo, we analyzed the cell cycle status in the different immune tissues of noninfected animals and at 7 days after infection by assessing the cellular DNA status along with eGFP expression (Fig. 4A). In noninfected animals, around 10% of bone marrow cells were in division, followed by 5% of thymocytes, while fewer than 1% of cells in spleen, lymph node, and PBMCs were actively dividing (Fig. 4B, left panels). At 7 days after infection, the cell cycle status in the bone marrow, lymph node, and PBMCs remained unchanged, while the percentage of dividing cells in the thymus dropped by 50%, and the rate of division in the spleen increased 10-fold (Fig. 4B, right panels). In all tissues, the ratio between infected and noninfected cells remained constant across the different cell cycle stages.

Infection does not affect overall apoptosis levels in immune tissues. To investigate the overall incidence of apoptosis during infection, we determined the proportion of AnV- and TUNEL-positive cells at the different time points. AnV detects externalized phosphatidylserine, which is indicative of early apoptosis, while TUNEL staining identifies DNA fragmentation associated with late apoptosis. Dead cells were excluded from AnV analyses by costaining with 7-AAD (Fig. 5A). The percentage of cells undergoing phosphatidylserine externalization remained stable in all tissues except the spleen, where a significant decrease ($P < 0.05$) was observed at 7 days after the infection (Fig. 5B). Similar results were obtained when cells were analyzed by TUNEL assay. Since cells in late apoptosis are rapidly phagocytosed, levels were generally low, never exceeding 4% throughout the experiment (Fig. 5B). In spleen and PBMCs a gradual but incremental increase in TUNEL-positive cells was observed as infection progressed, while the levels in bone marrow, thymus, and lymph node remained stable.

The extent of early apoptosis in infected cells upon cultivation is tissue dependent. To determine if infected cells were more susceptible to apoptosis, the distribution of AnV-positive cells between infected and noninfected populations was analyzed. In thymus and lymph node, where more than 60% of cells were infected on day seven (Fig. 3A, left panels), close to 50% of the remaining noninfected cells were AnV positive, compared to only 15% of the infected population ($P < 0.05$) (Fig. 6). The opposite distribution was observed in the spleen, where of the 5% infected cells almost half became AnV positive, while the proportion of noninfected cells reached only...
15% ($P < 0.05$). Finally, the AnV distribution in bone marrow and PBMCs was proportional (Fig. 6).

**Infected immune cells are resistant to apoptosis progression.** In vivo, apoptotic cells are rapidly removed by natural clearance mechanisms (11), making it difficult to evaluate the extent of protection conferred by CDV. Thus, we took advantage of the fact that once in culture without stimulation, immune cells spontaneously enter into apoptosis. Since infection levels in spleen and bone marrow were low, the analysis focused on the PBMCs, thymus, and lymph node. The overall...
FIG. 4. Cell cycle status in the different tissues before and 7 days after infection. (A) The cell cycle status was determined by DNA quantification using propidium iodide staining (left panel) and expressed versus eGFP expression (right panel), allowing the discrimination of infected and noninfected cells. (B) Cell cycle versus infection status in bone marrow (BM), spleen, PBMCs, thymus, and mesenteric lymph node (LN) from noninfected control ferrets and 7 days after infection with 5804PeH. The percentage of cells in each quadrant is indicated in the corners of the dot plot. Data are representative of three animals per time point.

FIG. 5. Proportions of early and late apoptosis in immune tissues during the first week after infection. Cells in early apoptosis were identified by AnV staining in conjunction with 7-AAD staining in order to exclude dead cells; cells in late stages of apoptosis were identified by TUNEL assay. Samples were analyzed by flow cytometry. (A) Typical dot plot of AnV/7-AAD staining of ferret thymocytes. The highlighted lower quadrants represent the populations included in subsequent analyses. (B) Percentage of cells at early (AnV+) or late (TUNEL+) stages of apoptosis in bone marrow (BM), spleen, PBMCs, thymus, and mesenteric lymph node (LN) at 0, 3, 5, and 7 days postinfection. The asterisk indicates a significant difference compared to noninfected control animals ($P \leq 0.05$).
percentage of AnV-positive cells increased 25 to 50% after 18 h in culture (compare Fig. 6 and 7A, left panels). In thymus and lymph node, and to a lesser extent in PBMCs, the majority of these cells were infected, leveling the proportions of infected and noninfected cells (compare Fig. 6 and 7A, right panels). In contrast, the overall incidence of TUNEL-positive cells increased up to fivefold in cells from noninfected animals and more than eightfold in samples from day 7 (compare Fig. 5B and 7B, left panels). However, most of these TUNEL-positive cells were noninfected (P < 0.05) (Fig. 7B, right panels). Taken together, these results indicate that CDV infection selectively inhibits the transition from early to late apoptosis in infected cells, while promoting this transition in noninfected cells.

**DISCUSSION**

Induction of apoptosis in response to virus infection is an effective antiviral immune response, as it not only impedes virus replication but also stimulates the clearance of infected cells by phagocytosis, thus enhancing the development of a specific adaptive immune response (6). In the case of morbilliviruses, infection-induced apoptosis of immune cells has been proposed as a main factor contributing to the observed leukopenia and the resulting immune suppression (10). There is experimental evidence for apoptosis induction, either directly in infected cells or as a bystander effect in noninfected cells (18, 25, 41), as well as for inhibition of apoptosis in the context of viral interference with the host response on a cellular level (7, 37). To gain better insight in the extent of apoptosis in immune tissues during the crucial early stages of a natural infection, we assessed levels of early and late apoptosis at different time points during the first week after infection in ferrets infected with a wild-type CDV strain.

**CDV-infected cells resist chemically induced apoptosis in vitro.** Infection of MDCK cells, which represent one of the main morbillivirus host cell types, resulted only in a slight increase in the proportion of cells experiencing DNA fragmentation and caspase 3 activation. Further examination revealed that apoptotic cells in these cultures were mainly noninfected and that infected cells were able to resist chemically induced apoptosis, indicating that CDV infection had a predominantly antiapoptotic effect. The discordance with other in vitro studies that reported CDV- or MeV-induced apoptosis in different cell lines might be due to the fact that the cells used did not originate from a host species or to differential alterations in the pro- and antiapoptotic pathways associated with their immortalization (10, 16, 18). Our findings support recent reports of MeV V and C protein-mediated interference with the key apoptosis inducers PKR and p73 (7, 37) and are consistent with the observed antiapoptotic activity of other members of the Paramyxoviridae family (35, 34, 42).

**What are the causes of morbillivirus-associated leukopenia?** Ferrets infected with 5804PeH, the strain used in this study, succumb to the disease within 2 weeks after intranasal infection (38). Even though these animals experience a more extreme leukopenia and higher infection levels in lymphocytes than are seen in MeV-infected patients or primates (2, 8, 25), the extents of early and late apoptosis as well as overall viability in cells isolated from different immune tissues remained generally stable. Consistent with reports from MeV patients and experimentally CDV-infected dogs (25, 31), we observed a gradual increase in early and late apoptosis in PBMCs. However, it is unlikely that an increase of early apoptosis from 15% to 22% and of late apoptosis from 3% to 5% solely accounts for the loss of more than 90% of PBMCs observed in this model during the same period.

Insufficient leukocyte production and maturation due to cell

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**FIG. 6.** Proportions of infected and noninfected cells in early apoptosis in the different tissues. The percentages of early apoptotic cells in infected and noninfected populations were assessed by flow cytometry using AnV and eGFP as markers for early apoptotic and infected cells, respectively. Cell suspensions of bone marrow (BM), spleen, PBMCs, thymus, and mesenteric lymph node (LN) were analyzed before and 7 days after infection (left panels). The means and standard errors (n = 3) of early apoptosis (AnV+) in infected and noninfected cells at 7 days postinfection are plotted in the respective right panels. Asterisks indicate a significant difference between infected and noninfected cells (P < 0.05).
cycle arrest has also been identified as a factor contributing to MeV leukopenia (22, 23, 30). Our steady-state analysis of the different tissues indeed revealed a 50% reduction of dividing cells in the thymus, at a time when more than 80% of the cells were infected. However, this cell cycle arrest did not translate into an increase in apoptotic cells, suggesting that CDV, like MeV (9), disrupts the regulation of cellular homeostasis. In contrast, division rates in the bone marrow and spleen, where infection levels remained below 10%, were stable or even increased considerably. Even though we cannot provide a definite explanation for this 10-fold increase in cell division in the spleen, it is more likely to be a consequence of the infection-induced destruction of the splenic architecture (38) than the result of extramedullary hematopoiesis. Taken together, our findings indicate that the disruption of white blood cell production alone may also not explain the infection-induced leukopenia. Instead, the massive loss of blood leukocytes within the first 5 days when infection levels are still low suggests that redistribution to sites of infection such as immune and epithelial tissues may be an important contributing factor.

**Inhibition of apoptosis progression in infected immune cells: a mechanism of immune evasion?**  The induction of apoptosis in response to infection is an integral part of the innate immune response. Apoptotic cells are rapidly phagocytosed, leading to the presentation of viral antigens and the development of an appropriate adaptive immune response (1, 11). For morbilliviruses, pro- and antiapoptotic effects have been observed in infected cells (7, 10, 37), suggesting that the ultimate outcome may be tissue and disease stage dependent. In addition, a bystander effect on noninfected cells has been observed in MeV patients (25) as well as experimentally both in vitro and in rodent models (19, 23, 24, 32), and several underlying mechanisms have been proposed: exposure to MeV envelope proteins was shown to be sufficient to inhibit proliferation of naïve PBMCs (28), and another study reported that nucleocapsid protein released from infected human thymic epithelial cells inhibits thymocyte proliferation and activates apoptotic pathways (20), all of which will ultimately lead to cell death of uninfected cells. Our study clearly documents that CDV infection renders immune cells more resistant to apoptosis progression, while increasing levels of apoptosis in noninfected cells in the same tissue, thereby reproducing the MeV bystander effect. It is unclear if the AnV-positive infected cells ultimately die by progressing to late apoptosis, necrosis, or virus-mediated disintegration of the cellular membrane in the context of syncytium formation. However, inefficient antigen presentation due to the delayed cell death of infected cells combined with an inability of the remaining uninfected cells to respond...
due to the bystander effect may contribute importantly to morbillivirus-induced immune suppression.

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