Receptor (SLAM [CD150]) Recognition and the V Protein Sustain Swift Lymphocyte-Based Invasion of Mucosal Tissue and Lymphatic Organs by a Morbillivirus

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Experimental infections of ferrets with canine distemper virus (CDV) recapitulate many hallmarks of measles: rash, high fever, viremia, depression of delayed-type hypersensitivity responses, lowered leukocyte counts, and reduced lymphocyte proliferation activity. To understand how a morbillivirus invades the host and causes immunosuppression, we generated CDV either unable to recognize one of the receptors or incapable of expressing either one or both of the candidate interferon antagonist proteins V and C. Variants of these viruses expressing green fluorescent protein were also generated. Striking similarities between CDV infection of ferrets and human immunodeficiency virus host invasion were documented: first, massive early replication in the gut-associated lymphatic tissue, including intestinal Peyer’s patches, followed by extensive infection of lymphatic organs, including thymus and circulating lymphocytes. Moreover, T cells were selectively depleted. Thus, CDV takes advantage of mucosal surfaces for host invasion and lymphocytes for swift dissemination. A CDV unable to recognize the signaling lymphocytic activation molecule (SLAM [CD150]) that is expressed in lymphocytes and other immune cells did not spread. A V-defective CDV multiplied with reduced efficiency in lymphocytes and did not inhibit the interferon and cytokine responses. Protein C affected the severity of rash and digestive symptoms elicited by V-defective CDV, but it was dispensable for the invasion of the lymphatic organs. These findings prove formally that SLAM recognition is necessary for morbillivirus virulence. They also reveal how two viral proteins affect pathogenesis: V sustains the swift lymphocyte-based invasion of mucosal tissue and lymphatic organs, whereas C sustains subsequent infection phases.

Measles virus (MV) infection is a major cause of child mortality that is most often due to severe suppression of immune responses (17, 28, 38, 55). Therefore MV, the human member of the Morbillivirus genus, has been targeted by the World Health Organization for eradication. Animal morbilliviruses are also important pathogens; these include a virus that has a major economic impact on domestic livestock (rinderpest) and one that has a broad and expanding host range in small carnivores (canine distemper virus [CDV]) (18, 36). All of these viruses have 16-kb, nonsegmented, negative-strand RNA genomes with six genes arranged in nonoverlapping transcription units. One of these genes codes for three proteins, a polymerase cofactor (phosphoprotein [P]) and two candidate interferon antagonists, V and C. All of the other genes code for a single product.

The lack of a small animal model has limited studies of morbillivirus immunosuppression. Mice expressing the two candidate primary MV receptors membrane cofactor protein (CD46) or signaling lymphocytic activation molecule (SLAM [CD150]) have been used to study MV host invasion (29, 33, 41, 56), even if no classical immunosuppression symptoms were reported. Thus, macaques remain the best model for measles (3, 57); however, these primates are expensive and in short supply. Not being in the position of designing a completely humanized mouse to study measles pathogenesis, we developed reverse genetics for CDV, a morbillivirus that naturally infects a laboratory animal, the ferret (50, 53).

CDV infection of ferrets recapitulates many MV-induced immunosuppression hallmarks: depression of tuberculin/delayed-type hypersensitivity (DTH) test responses, lowered leukocyte counts and antibody titers, and reduced in vitro lymphocyte proliferation activity. CDV-infected ferrets develop rash, high fever, and viremia and die within 2 to 3 weeks of intranasal inoculation. CDV infection of ferrets has consequences similar to those of rinderpest infection of Bovidae (5) and is more severe than MV infection of humans or other primates, appearing ideal to characterize pathogenic mechanisms. Studies of the morbilliviruses have identified three shared candidate pathogenesis determinants: SLAM recognition (46) and two candidate interferon antagonist proteins.

The nature of the principal morbillivirus receptor has been contentious: the ubiquitous regulator of complement activation CD46 can act as a port of cell entry for MV of the vaccine lineage (12, 31), but wild-type as well as vaccine-lineage MV strains also interact with SLAM (13, 21, 45), an immune cell-specific protein. SLAM is constitutively expressed on immature thymocytes, CD45RO(high) memory T cells, and a proportion of B cells; it is rapidly induced on a wide range of immune cells, including T and B cells after activation, and is involved in immune regulation (9, 42, 49).

The observations that different morbilliviruses use SLAM as a cellular receptor (4, 46), while human CD46 may interact
with only vaccine-lineage MV (15), are consistent with a central role for SLAM in morbillivirus virulence. To generate a CDV incapable of recognizing SLAM while maintaining entry into epithelial cells, we mutagenized the CDV hemagglutinin (H), identified residues that sustain SLAM-dependent cell fusion, and combined them in an infectious CDV cDNA (52). This SLAM-blind (SLAMblind) CDV infected primary ferret epithelial cells as efficiently as did the parental wild-type 5804P strain but was incapable of entering ferret peripheral blood mononuclear cells (PBMC). Here we assay the virulence of the SLAMblind CDV in ferrets.

Viral pathogenesis is also influenced by intracellular events downstream of receptor binding, in particular by viral proteins counteracting host innate immune responses. Morbilliviruses and other members of the subfamily Paramyxovirinae inhibit interferon signaling using diverse strategies. These strategies always emanate from the P gene and involve interactions with STAT proteins (1, 16, 32, 34, 40, 47). In certain Paramyxovirinae, the P gene gives rise to many polypeptides (24) but, in morbilliviruses, P codes for only P, V, and C, simplifying the analysis of potential virulence determinants. The V and C proteins are dispensable for viral growth in cultivated cells but necessary for host invasion (30). They have multiple functions: for example, the Sendai virus C protein facilitates particle assembly (37).

To assess the relevance of the V and C proteins for host invasion and pathogenesis, we produced (by reverse genetics) viruses in which these proteins were inactivated: C knockout (Cko), V knockout (Vko), and V and C knockout (VCko) CDV. We here document how the V protein fuels an infection that is lymphocyte based and blazes through the mucosal tissue and lymphatic organs. The C protein has a more subtle effect on subsequent infection phases. This work sheds new light on the role of the proteins previously known as “accessory” for morbillivirus pathogenesis.

**MATERIALS AND METHODS**

**Cells and viruses.** VerodogSLAMtag cells (53), primary ferret epithelial cells (52), and 293 cells (ATCC CRL-1573) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 5% fetal calf serum (Invitrogen). One mg/ml Zeocin (Invitrogen) was added to the VerodogSLAMtag cells to maintain selection of canine SLAM expression. Aside from the SLAMblind strain (52) that was propagated on primary ferret epithelial cells, all other viruses were grown on VerodogSLAMtag cells.

**Generation of recombinant viruses.** The combination of mutations described below and in the legend for Fig. 1A were introduced in a subcloned P gene fragment flanked by the unique restriction sites PshAI and SalI and transferred into the 5804P genomic cDNA using these restriction sites (53). To construct derivatives of Vko and Cko expressing green fluorescent protein (GFP), the mutated P genes were transferred to the 5804P*green* genomic cDNA (50). Recombinant viruses were recovered using a reverse genetics system based on B cells and expanded on VerodogSLAMtag cells basically as outlined previously (53). Kinetic analyses of virus growth were performed as described previously (54).

**CDV V- and C-specific antisera.** To assess V and C protein expression, we raised (in rabbits) antisera against two peptides: SAKGWNASKPSERILC corresponding to amino acids 1 to 16 of the C protein, and ALRENPPDIEEIQEVSLLRDQ(T)C, corresponding to amino acids 18 to 39 of the P and V proteins with an added carboxyl-terminal cysteine. Methods for antibody production and immunoblotting were as described previously (51).

**Animal experiments and assessment of virulence.** Experiments with unvaccinated male ferrets (Mustela putoris furo) were performed as described previously.
were measured after migration in a 1% agarose gel using the Chemigenius2 1 min at 60°C and 10 s at 94°C. Optical densities of the resulting PCR products consisted of a denaturation step of 2 min at 94°C, followed by 35 cycles between Taq l reaction mixture using the respective gene-specific primers amplified in a 10- Superscript RT-III (Invitrogen, Carlsbad, CA) and the different genes were PCR time point, 100 ng RNA was reverse transcribed using an oligo(T) primer and (QIAGEN, Valencia, CA) and quantified by UV spectrophotometry. For each parameter, the maximum grade was defined as that induced by the fully virulent CDV strain 5804P (53), the intermediate grade reflected the virulence of the attenuated strain 5804 (53), and the lowest grade corresponded to no or minimal immunosuppression.

The threshold values used (0, 1, and 2) were as follows: leukocyte number, always above 5,000/mm³, always above 2,000/mm³, and at least once below 2,000/mm³. Lymphocyte proliferation activity, always close to initial activity, three or more values below 80% initial activity, and one or more values below 25% initial activity; neutralizing antibody titers, above 100 at week 2, above 100 at week 3 or later, and never above 100; viremia (cell-associated), none, cleared after 3 weeks, and positive for more than 3 weeks or at death; and DTH response, always positive, temporarily negative, and negative at death.

Viremia was quantified by measuring the fraction of infected PBMC. PBMC were separated from erythrocytes in EDTA-treated blood by lysing the red cells in ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.01 mM EDTA, pH 7.2 to 7.4), counted, and cocultivated with VerodogSLAMtag cells in quadruplicates of 10-fold dilutions. Wells were evaluated for syncytia formation after 3 days, and results were expressed as tissue culture infectious doses per 10⁶ cells. The remaining cells were resuspended in RNAAlater (QIAGEN, Valencia, CA) and stored at ~20°C. Fluorescence-activated cell sorter (FACS) analysis and imaging were basically as described previously (50).

Cytokine mRNA: cloning of cDNAs and quantification. Since cDNAs from ferret cytokines were not available, to be in the position of quantifying expression of their mRNAs, we cloned them for this, oligonucleotide primers were synthesized based on conserved regions of the homologous canine and feline genes. These primers were used to reverse transcribe and amplify mRNA from phytohemagglutinin-stimulated ferret PBMC. The amplified fragments were sequenced, and other sets of primers entirely homologous to the ferret sequences were synthesized. The homologous primers were used for semiquantitative reverse transcription-PCR. The sequences of these primers are available on request.

To quantify changes in cytokine mRNA expression levels, RNA from ferret PBMC stored at −20°C in RNAAlater was isolated using the RNAeasy mini kit (QIAGEN, Valencia, CA) and quantified by UV spectrophotometry. For each time point, 100 ng RNA was reverse transcribed using an oligo(T) primer and Superscript RT-III (Invitrogen, Carlsbad, CA) and the different genes were PCR amplified in a 10-µl reaction mixture using the respective gene-specific primers in combination with Taq polymerase (NEB, Ipswich, MA). The melting temperature of all primers was close to 60°C, allowing the use of a two-step protocol that consisted of a denaturation step of 2 min at 94°C, followed by 35 cycles between 1 min at 60°C and 10 s at 94°C. Optical densities of the resulting PCR products were measured after migration in a 1% agarose gel using the Chemigenius2 XE imaging system and analyzed with the accompanying GeneTools software (Syngene, Frederick, MD). The relative amount of cytokine mRNA was expressed as the ratio of cytokine to the housekeeping gene aldolase, and the upregulation of gene expression was calculated as an increase (n-fold) relative to the cytokine/aldolase ratio of the respective animal prior to infection. Final values for each cytokine and time point were calculated as the means from two separate amplifications based on different cDNA syntheses, and a third reaction was added if the two values differed by more than 15%.

RESULTS

Recombinant viruses that do not express the candidate interferon antagonists. We generated recombinant CDV unable to express the V and C proteins, both encoded by the P gene. V has a cysteine-rich, zinc-binding, RING-finger-type domain accessed by RNA editing (Fig. 1A) (8). C is encoded in an alternative reading frame accessed by ribosomal frameshifting (Fig. 1A) (6). We introduced into the P gene combinations of mutations interfering with RNA editing, C protein translation initiation, or both as well as early stop codons in the appropriate reading frames (Fig. 1A, bottom panel). These mutations did not interfere with P protein expression, and all three viruses (Cko, Vko, and VCko) were rescued. They replicated efficiently in Vero cells, producing the expected sets of proteins as documented in Fig. 1B. The kinetics of intracellular virus production (Fig. 1C) and particle release (Fig. 1D) were also analyzed. Cko growth kinetics were equivalent to those of wild-type 5804P, whereas Vko and VCko grew to slightly lower titers (Fig. 1D). We also previously generated and characterized a selectively receptor-blind CDV (52); the sequence of the mutated segment of its H gene is shown in Fig. 1A, top panel. The Vko, Cko, and VCko viruses retain SLAM binding.

A SLAMblind CDV is completely attenuated. We inoculated groups of four ferrets intranasally with 10⁷ infectious units of the SLAMblind, Vko, Cko, and VCko viruses. We scored virulence and immunosuppressive activity of these viruses and compared these results with those previously published for infections of four ferrets with the parental wild-type strain 5804P (53). The extent of rash, duration of fever, and onset and severity of diarrhea were measured; values measured for individual ferrets are reported by the shades of the squares (Fig. 2A). The average severity of these symptoms is expressed as the virulence index (Fig. 2A). White blood cell count, in vitro lymphocyte proliferation activity, neutralizing antibody titers, cell-associated viremia, and DTH response were also assessed, and an immunosuppression index was calculated based on the average magnitude of these parameters (Fig. 2B).

The means and standard deviations of the results obtained in each animal’s group are indicated in Fig. 2D through F for selected parameters. The SLAMblind virus was completely avirulent in four ferrets (each animal is represented in Fig. 2A, second row); infection with this virus caused a small but statistically significant, short-lived decrease in blood leukocyte count (Fig. 2E). Nevertheless, this virus induced protecting levels of neutralizing antibodies, which appeared with slow kinetics (Fig. 2B, third column, and data not shown), providing evidence that it replicates at a minimal level in vivo. In contrast, SLAMblind replicates with kinetics equivalent to those of the parental strain in primary ferret epithelial cells (52).

A C-defective CDV is fully immunosuppressive. The Cko virus replicated efficiently and was as virulent and immunosuppressive as wild-type 5804P (Fig. 2A and B, compare first and third rows, and Fig. 2C to F; compare results for 5804P and Cko). All animals infected with Cko succumbed to the disease with kinetics equivalent to those of animals infected with 5804P (Fig. 2C) and had similar levels of viremia (Fig. 2D).

To reveal the sites of preferential viral replication in the host, we previously generated a wild-type derivative expressing

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GFP (5804Pgreen) (50). Similarly, we generated a Cko green derivative expressing GFP from an additional transcription unit inserted after the H gene. Seven days postinoculation (p.i.) all of the lymphatic organs of hosts infected with Ckogreen emitted green fluorescence. Fluorescence was particularly intense in the intestinal Peyer’s patches (Fig. 3B and C). The intensities of the signals were similar to those documented for 5804P at this early time point (50), demonstrating strong replication in the gut-associated lymphatic tissue. Seven days p.i., fluorescence was also very strong in the thymus (Fig. 3H and I), similar to previous observations with the wild-type derivative 5804Pgreen (50).

To assess whether mutations selected during replication resulted in reversion to C protein expression, we isolated CDV from PBMC of two Cko-infected ferrets and verified their protein expression profile and P gene sequences. Since no revertants were selected (data not shown), we conclude that the C protein is not essential for CDV-elicited immunosuppression in ferrets.

A V-defective CDV is attenuated. The Vko virus caused only mild signs of disease (Fig. 2A, fourth row), was mildly immunosuppressive (Fig. 2B, fourth row), and caused limited viremia (Fig. 2D), and all infected animals survived (Fig. 2C). Vko caused transient leukopenia, but the leukocyte number re-
bounded 2 weeks p.i. and reached normal levels 4 to 5 weeks p.i. (Fig. 2E). Moreover, Vko was incapable of inhibiting lymphocyte proliferation (Fig. 2F). The Vko virulence index (1.4) was intermediate between full virulence (3.0) and complete attenuation (0), reflecting intermediate values for all three parameters, rash, fever, and diarrhea.

To visualize the spread of a V-defective virus, we constructed a Vko\textsuperscript{green} derivative expressing GFP, infected ferrets, and monitored green light emission in animals that were euthanized 7 days p.i. In Vko\textsuperscript{green}-infected hosts, fluorescence was reduced compared to that in Cko\textsuperscript{green} infected hosts. Fluorescence was only slightly greater than background levels in the thymus (Fig. 3K and L). The strongest fluorescence was observed in segments of the intestine and had a pattern characteristic of Peyer’s patches (Fig. 3E and F). Thus, gut-associated lymphatic tissue may be the major site of viral multiplication at early infection times.

The C protein sustains the rash and digestive symptoms in a V-defective background. The virus defective for both V and C expression (VCko) had an immunosuppression index very similar to that of Vko (0.6 versus 0.7, respectively) (Fig. 2D, fifth and fourth rows, respectively), reflecting equivalent levels of viremia, leukopenia, and lymphocyte proliferation inhibition (Fig. 2D to F, compare results for VCko and Vko). However, an attenuating effect of C protein ablation was documented, as evidenced by a virulence index that was 0.5 for VCko and 1.4 for Vko (Fig. 2A, fifth and fourth rows, respectively). This difference was due to the absence of rash and diarrhea in VCko-infected hosts.

Leukopenia is associated with virus spread that is extensive only upon SLAM recognition. All viruses recognizing SLAM caused a significant drop in leukocyte number, which was halved 3 days p.i. and halved again 7 days p.i. (Fig. 2E), suggesting emigration of these short-lived cells from the blood, probably to sites of infection and inflammation. The fact that Vko and VCko induced a steep drop in leukocyte numbers equivalent to that of the wild-type or Cko is consistent with initial productive infection by these viruses. Transient and mild leukopenia in SLAMblind-infected animals (Fig. 2E) is consistent with SLAM recognition being essential for robust replication in the initial infection phase.

Viral load in PBMC correlates with virus spread in lymphatic organs. Having shown that infection of the lymphoid organs by CDV is rapid and massive, we assessed viral load in

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FIG. 3. Replication of Cko\textsuperscript{green} and Vko\textsuperscript{green} in thymus and intestinal Peyer’s patches. Visualization of infection in Peyer’s patches or thymus in a typical infection of hosts inoculated with Cko\textsuperscript{green} (A to C and G to I) or Vko\textsuperscript{green} (D to F and J to L). (A and D) Photographs of the peritoneal cavity. (G and J) Photographs of the thymus, which contours an outlined white line. (B, E, H, and K) Same organs as above, but photographed after GFP fluorescence excitation. (C, F, I, and L) Microscopic analysis of the same tissues as the second-row panels.
PBMC 3 to 14 days p.i. (Fig. 4A). In animals inoculated intranasally with the virulent strain 5804P or Cko, no infected PBMC were detected 3 days p.i. However, 7 to 14 days p.i., 40 to 50% of PBMC were infected (Fig. 4A). Vko-inoculated animals demonstrated similar kinetics of infection, but the fraction of infected cells was 6 to 20 times reduced (Fig. 4A) and cell-associated viremia was about 100-fold reduced relative to the wild-type or Cko (Fig. 2D, compare results for Vko with results for 5804P). PBMC infection was below detection levels in SLAMblind-infected animals (Fig. 2D). Thus, viral load in PBMC correlates with the extent of virus spread to lymphatic organs; virus is detected first in lymphatic organs and later in blood.

V sustains viral replication in lymphocytes and T-cell depletion. To gain insight into the cell types supporting viral spread, we analyzed virus replication separately for B cells, T cells, and monocytes. We used pan-species antibodies that cross-react with ferret CD3 (T cells), CD79α (B cells), or CD14 (cells of the monocytic lineage). Figure 4B presents representative FACS data obtained 7 days p.i. from two ferrets. One animal was infected with wild-type CDV (5804P, upper row) and the other with Vko (lower row). Figure 4C and D present the average measurements from groups of three animals infected either with 5804P or with Vko.

About half (30 to 60%) of the T and B cells of the ferrets inoculated with 5804P were infected 7 to 14 days p.i. (Fig. 4B, top row, second and third panels, compare upper left and right quadrants; and Fig. 4C, compare lengths of the CDV- and CDV+ segments of columns). Infection with 5804P resulted in selective T-cell depletion: T cells represented 60% of total PBMC at 0 to 7 days p.i., about 40% 10 days p.i., and less than 40% 14 days p.i. shortly before the animals were euthanized (Fig. 4C, add lengths of the CDV- and CDV+ segments of the columns). Infection of B lymphocytes by 5804P was similarly efficient (Fig. 4C) but did not cause their depletion. In the context of the leukopenia documented at day 7, we conclude that the increase in B-cell percentage reflects T lymphocyte depletion.

Vko infected the same cell types as 5804P did but with 5 to 10 times reduced efficiency (Fig. 4B, bottom row, second and third panel, upper quadrants; and Fig. 4D). The V-defective virus did not deplete the T-cell fraction of PBMC (Fig. 4D, Vko D7 segments in the bars). Thus, V sustains massive lymphocyte infection and their subsequent depletion.

Cytokine responses are depressed in wild-type but not in Vko infections. Since the paramyxoviral V protein inhibits interferon signaling (10, 20), we compared the expression of alpha interferon and five other cytokines in hosts infected with wild-type and Vko CDV. Since antibodies recognizing ferret
cytokines are not available, we cloned their cDNAs and established reverse transcription-PCR assays to measure the levels of the respective mRNAs in PBMC. We then inoculated ferrets with wild-type or Vko CDV, obtained PBMC 3 and 7 days p.i., and measured the cytokine response.

In animals infected with wild-type virus, levels of expression of most cytokines remained close to those monitored before infection (Fig. 4E). At days 3 and 7, we observed minimal increases or decreases in levels of tumor necrosis factor alpha and interleukin-6 (IL-6), two early-response cytokines secreted by phagocytes. The expression of the T-cell cytokine IL-2, which is responsible for T-cell growth, was elevated only on day 3. The expression of gamma interferon, another T-cell cytokine that controls macrophage activation, remained below the pre-infection level at both time points. The expression of a third T-cell cytokine, IL-4, which activates B cells and promotes the immunoglobulin M (IgM)-IgG class switch, was slightly elevated at day 3. Thus, phagocytes and T cells of animals infected with 5804P were unable to express several of the cytokines that orchestrate infection control and induce the IgM-IgG class switch required for an efficient humoral response. In addition, alpha interferon production did not increase.

In contrast, at day 3, Vko triggered upregulation of the two immediate response cytokines tumor necrosis factor alpha and IL-6. The levels of alpha interferon were also high, as were the levels of gamma interferon and IL-2, indicative of a Th1-dominated early response. IL-4 induction coincided with the onset of a neutralizing antibody response on day 7 (Fig. 4E). These observations indicate that an effective antiviral cytokine response is in place in animals infected with Vko. Thus, the V protein is instrumental for sustaining efficient viral replication in T cells, inhibiting expression of not only alpha interferon but also several cytokines, and inactivating the adaptive immune response.

**DISCUSSION**

**Mechanisms of host invasion and immunosuppression in morbillivirus and HIV infections.** This study revealed striking similarities between the basic mechanisms of CDV host invasion and those sustaining acute phases of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection. First, massive HIV and SIV replication occurs early in lymphatic organs, including the gastrointestinal mucosal tissue (7, 26, 48). Thus, acute disease in a measles model based on intranasal inoculation, an AIDS model based on intravaginal inoculation, and probably measles and AIDS is characterized by swift and massive viral replication in lymphatic organs and mucosal tissue. SIV dissemination is also rapid following oral inoculation, reflecting infection of the oral and esophageal mucosa as well as tonsils (27). CDV spreads rapidly in the tonsils after experimental intranasal infection (2). Altogether, these observations suggest that morbillviruses and primate lentiviruses can take advantage of any mucosal surface to enter the host and rapidly disseminate.

A second similarity between CDV and human lentivirus host invasion is massive lymphocyte infection followed by T-cell depletion. One week after inoculation, about half the lymphocytes circulating in peripheral blood or residing in lymphoid organs, including the thymus, spleen, and lymph nodes, are CDV positive (50). Similar levels of SIV-positive cells have been recently documented in the acute phase of infection (25). These levels of infection imply that the hosts may lose most T cells and suggest that virally mediated destruction is the main cause of their depletion. Nevertheless, bystander effects may be important in subsequent infection phases.

Based on the inability to detect robust viral replication in the PBMC of measles patients, immunosuppression mechanisms more complex than direct destruction and depletion of lymphocytes have been put forward (28, 38). We think that MV replication in human lymphocytes may have been underestimated. In experimental infections of macaques, MV replication is extensive about 1 week after inoculation, when up to 10% of the PBMC (57) as well as 3 to 15% of cells in lymph nodes, spleen, and thymus (44, 57) are infected. Similarly, when the PBMCs of patients with measles are examined early after rash, up to 5% of infected cells can be detected, whereas at later time points, the fraction of infected cells drops (14). MV replication in PBMC may peak at the onset of rash, before clinical samples are taken.

**V is the essential interferon antagonist and cytokine response inhibitor.** We show here that the CDV V protein is essential for rapid viral multiplication in T cells. On the other hand, the Sendai virus V protein sustains viral spread in the airway epithelium. In this tissue, replication of a V-deficient Sendai virus is efficient for 1 to 2 days but then infection is rapidly cleared (22). Similarly, the V-deficient CDV spreads in the lymphatic organs but infection is cleared.

These effects are probably due to the interferon antagonist and cytokine response inhibitor function of V: we have shown that the V-protein expressing CDV almost completely inhibits induction of alpha interferon and five other cytokines, and it is known that the V proteins of most paramyxoviruses inhibit interferon signaling and also interferon transcription. These proteins associate with STAT1/STAT2 complexes and abolish interferon JAK/STAT signaling by targeting either STAT1 or STAT2 for proteasomal degradation or by interfering with STAT phosphorylation, as reviewed in references 10 and 20. In addition, they interfere directly with beta interferon transcription (19, 35).

As to the mechanisms of inhibition of the other cytokines, it was recently shown that Sendai virus V binds the cytoplasmic interferon-inducible RNA helicase mda-5 (1). Mda-5 or the other helicase RIG-I then contacts an adaptor named IPS-1 (beta interferon promoter stimulator 1) (23) or MAVS (mitochondrial antiviral signaling) (39) that activates NF-κB and IRF3. Thus, the V protein of Sendai virus controls an essential pathway in the regulation not only of the beta interferon promoter but also of other cytokines and of innate immunity. The V protein of morbilliviruses may have an equivalent function.

**C is an infectivity factor.** Lack of C protein expression resulted in reduced virulence in the V-defective genetic background. On the other hand, the CDV that was only C-defective remained fully virulent and immunosuppressive. This was surprising in view of the multiple functions of the MV and Sendai virus C proteins and since these proteins are necessary for virulence in natural hosts (30, 44). In particular, the phenotypes of a C-defective MV in macaques and a C-defective CDV in ferrets are different. In the lymphatic organs of macaques obtained 10 days after inoculation with wild-type MV, up to
15% of the cells were infected, whereas in macaques infected with a C-defective MV, about 100 times fewer cells were infected (44). Thus, C is necessary for MV spread in the lymphatic organs of macaques.

It is possible that the immune system of ferrets is defective for the protein interacting with CDV C. Nevertheless, the double-knockout, VC-defective CDV lost competence to induce rash and digestive symptoms in ferrets. Knowing that one of the functions of the morbillivirus C protein is to sustain the replication in the lymphatics is reduced at least 100 times in infections with the V-defective CDV, particle instability may become the limiting factor for spread to the epithelia. Thus, the rash and digestive symptoms do not develop. On the other hand, the C-defective virus may be ferried by lymphocytes through the lymphoid organs, allowing the retention of full immunosuppressive activity, which results in host death.

SLAM recognition and host invasion. Replication of the SLAMblind CDV was inferred from the detection of mild leukopenia and protective levels of neutralizing antibodies in two of four animals. However, it was not possible to identify and localize the sites of replication, even using a GFP-expressing virus (data not shown). Wild-type CDV replication was detected in epithelial cells of the respiratory tract only in late infection stages (50), suggesting that lung epithelium infection follows rather than precedes CDV amplification in lymphatic organs. This is consistent with the observation that MV preferentially enters well-differentiated human airway epithelia through the basolateral surface (43). Thus, morbilliviruses may invade epithelia only in late infection phases and from the basolateral surface.

This sequence of events is fundamentally different from that considered in a current MV infection model (17). This textbook model implies initial infection of epithelial cells in the respiratory tract through an unknown receptor, followed by secondary replication in local lymphatic tissues. In CDV infections of ferrets, the unknown epithelial cell receptor sustains lung and bladder invasion only when massive amounts of virus are produced from infected lymphocytes and thus may have lower affinity for H than does SLAM. We suggest that similar events may sustain the spread of wild-type MV in humans after contagion: these viruses may initially target SLAM-expressing lymphocytes or tissue macrophages, possibly situated at the bottom of the crypts in the tonsils, rather than an unknown epithelial cell receptor. To assess this new model, a systematic analysis of the early phases of MV infection in the buccal cavity and the upper respiratory tract of experimentally infected macaques, quantifying the extent of MV replication in epithelial and lymphatic cells, is necessary.

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