Severe Impairment of Dendritic Cell Allostimulatory Activity by Sendai Virus Vectors Is Overcome by Matrix Protein Gene Deletion


*J Immunol* 2005; 175:4971-4980; doi: 10.4049/jimmunol.175.8.4971

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Severe Impairment of Dendritic Cell Allostimulatory Activity by Sendai Virus Vectors Is Overcome by Matrix Protein Gene Deletion


Delivering of Ags to dendritic cells (DCs) plays a pivotal role in the induction of efficient immune responses ranging from immunity to tolerance. The observation that certain viral pathogens are able to infect DCs has led to a concept in which applications of recombinant viruses are used for Ag delivery with the potential benefit of inducing potent Ag-specific T cell responses directed against multiple epitopes. As a prerequisite for such an application, the infection of DCs by recombinant viruses should not interfere with their stimulatory capacity. In this context, we could show that an emerging negative-strand RNA viral vector system based on the Sendai virus (SeV) is able to efficiently infect monocyte-derived human DCs (moDCs). However, after infection with SeV wild type, both the response of DCs to bacterial LPS as a powerful mediator of DC maturation and the allostimulatory activity were severely impaired. Interestingly, using various recombinant SeV vectors that were devoid of single viral genes, we were able to identify the SeV matrix (M) protein as a key component in moDC functional impairment after viral infection. Consequently, use of M-deficient SeV vectors preserved the allostimulatory activity in infected moDCs despite an efficient expression of all other virally encoded genes, thereby identifying M-deficient vectors as a highly potent tool for the genetic manipulation of DCs.

cycle without any risk for chromosomal integration. Furthermore, SeV has not been linked to any disease in humans (19). These favorable basic vector characteristics, together with the recent observation that SeV is able to replicate nonproductively in bone marrow-derived mouse DCs, inducing DC maturation (20), suggested SeV as an interesting candidate for gene transfer approaches into human-derived DCs.

Until now, no purposeful infection of human DCs by SeV has been reported. In contrast, MeV, which constitutes a paramyxovirus closely related to SeV, is known to convey an efficient infection of human-derived DCs, followed by DC maturation, and also to induce a severe impairment of DC functions (21, 22). These features clearly exclude MeV as a possible vector for genetic DC manipulation, whereas comparable data on a possible interference with DC functions in the course of SeV infections are still missing.

Here, we show that SeV is able to efficiently infect monocyte-derived human DCs (moDCs). However, SeV wild-type infection rendered moDC insensitive to LPS-induced stimulation, induced DC apoptosis, and severely impaired the allostimulatory activity of moDCs. Interestingly, using various recombinant SeV vectors devoid of single viral genes, we were able to identify the SeV matrix (M) protein as a key component in moDC functional impairment after viral infection. Consequently, use of M-deficient SeV vectors preserved the allostimulatory activity in SeV-infected moDCs despite an efficient expression of all other virally encoded genes.

These findings identify M-deficient SeV vectors as a highly potent new tool for the genetic manipulation of DCs and suggest the possibility to use SeV vectors for the generation of Ag-specific T cell responses being directed against multiple epitopes.

Materials and Methods

Virus recovery and propagation

A recombinant SeV coding for the reporter gene gfp by an insertion between the leader sequence and the start of the viral n gene in the wild-type genome was grown in 9-day-old embryonated chicken eggs as described previously (23, 24). The generation and amplification of single gene-deleted SeV particles SeVΔM-gfp, SeVΔF-gfp, and SeVΔHN-gfp was performed as described recently (25).

Cell isolation and DC generation from adherent PBMCs

Generation of moDCs was performed as described previously (26). In brief, PBMCs were isolated by Ficoll-Paque (Life Technologies) density gradient centrifugation of heparinized blood obtained from buffy coat preparations of healthy volunteers from the blood bank of the University Clinic Tübingen. Cells were seeded (1 × 10^5 cells/ml) in 6-well plates (Costar) in RP10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin). After 2 h of incubation at 37°C, nonadherent cells were removed, and the adherent blood monocytes were cultured in RP10 media supplemented with the following cytokines: human recombinant GM-CSF (Leukomax, 100 ng/ml; Novartis Pharmaceuticals), IL-4 (1000 IU/ml; Genzyme), and for mature DCs with TNF-α (10 ng/ml; Genzyme). The phenotype of DCs was analyzed by flow cytometry after 7 days of culture.

Infection of moDCs

moDCs were washed once with X-VIVO 20 media (BioWhittaker). Infection with the indicated viral particles was performed either at a multiplicity of infection (MOI) of 1 or 2 in a total volume of 300 μl of X-VIVO 20. Cells were washed twice with RP10 media and cultured in RP10 media with supplements (see above). In the indicated experiments, 2 μg/ml bacterial LPS from Salmonella typhimurium (Sigma-Aldrich) were added for the last 24 h of culturing.

Immunostaining

Cell staining was performed using FITC- or PE-conjugated mouse mAbs against CD86, CD40 (BD Pharmingen), CD80, HLA-DR, CD14 (Becton Dickinson), CD83 (Coulter-Immunotech), and CD1a (OKT6; Ortho Diagnostics). Appropriate mouse IgG isotypes were used as controls (Becton Dickinson). The samples were analyzed on a FACSCalibur (BD Biosciences).

Nuclear extracts

moDC pellets were washed in 1 ml of ice-cold buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, and 1 mM DTT) and incubated for 10 min on ice in 1 ml of buffer A plus 0.4% Igepal CA-630 (Sigma-Aldrich). The cell membranes were centrifuged at 750 × g for 5 min. Pellets were resuspended in 200 μl of buffer B (20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM DTT), and nuclei were mechanically lysed for 2 h at 4°C. Cell debris were pelleted for 15 min at 7500 × g, and supernatant was recovered and stored at −70°C until use. Proteinase inhibitors (aprotinin and leupeptin; Sigma-Aldrich) were added to the buffers just before use.

PAGE and Western blotting for detection of ReIβ protein

Protein concentration of nuclear extracts were determined using a bicinchoninic acid assay (Pierce). Twenty micrograms of total protein were separated on a 12% polyacrylamide gel, blotted on a polyvinylidene difluoride membrane, and probed with a polyclonal rabbit ReIβ Ab (C-19; Santa Cruz Biotechnology) and a monoclonal mouse c-Rel Ab (B-6; Santa Cruz Biotechnology). Bands were visualized by ECL staining (Amersham Biosciences).

Detection of apoptosis

After the indicated time periods, moDCs were washed and fixed with 4% buffered paraformaldehyde (Merck). Leakage of fragmented DNA from apoptotic nuclei was measured as described previously (27). Cell nuclei were prepared by lysing cells in hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide) and immediately analyzed by flow cytometry (FACSCalibur). Nuclei containing hypodiploid DNA were considered to be apoptotic.

Mixed lymphocyte reaction

moDCs either infected or uninfected were cultured for 1 day. moDCs were washed, and different numbers were incubated with 10^5 allogeneic PBMCs in a total volume of 3 ml/well into 6-well plates (Costar) for 7 days, according to the manufacturer’s instructions.

Real-time quantitative RT-PCR

RNA was extracted using Nucleospin RNAII (Macherey-Nagel); contaminating DNA was digested according to the manufacturer’s protocol, and the remaining DNA was dissolved water and stored at −70°C. First-strand cDNA was prepared from total RNA by reverse transcription using Superscript RNAse H− Reverse Transcriptase (Invitrogen Life Technologies) and oligomers dT16−18 (specific for mRNA species) or the primer ld55 (specific for the leader region of the SeV genome; 5′-TAAAGTG CAAAGTATCCACCTGGAGAG-3′). Gene expression was measured in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR primer sets (Biomers.net) were selected with Primer software (Applied Biosystems) to result in ampiclons <450 bp to enhance efficiency of PCR amplification. The GFP primers were as follows: forward, 5′-TAAAGCG GCCAACAGTTCCAGCTGTT-3′; reverse, 5′-TTATTGACGCTGTCG-3′. The resulting PCR products were cloned directly into the vector pCR 2.1-TOPO by using the TOPO TA Cloning kit (Invitrogen Life Technologies) and subsequently sequenced to confirm primer specificity. The specificity of each PCR product was confirmed by agarose gel electrophoresis and dissociation curve analysis. Amplification of specimens and serial dilutions of the amplification standards was conducted in a total volume of 20 μl of SyberGreen Master Mix (Applied Biosystems) at optimized concentrations. Standard curves were generated for each gene, and the amplification was found to be 90−100% efficient. The threshold cycle (CT) at which the fluorescence passes...
the fixed threshold is inversely proportional with the starting amount of a specific RNA in the sample. Relative quantitation of gene expression was determined with the use of the comparative CT method as suggested by the manufacturer. All results are displayed relative to tubulin (differences between CT of GFP and tubulin (dCT)). Low dCT values denote a small difference between the signals of GFP and the housekeeping gene tubulin (i.e., high expression levels of GFP).

Statistical analysis
Two-way ANOVA with Bonferroni’s post hoc test was performed using GraphPad Prism version 4.00 (GraphPad). Significant difference was considered for p < 0.05.

Results
Immature and mature DCs are susceptible to SeV infection
A direct infection of moDCs by SeV has not yet been investigated. Therefore, SeV particles expressing 1) all viral genes (wild-type situation) together with the 2) GFP reporter gene (SeV-GFP) were used in different amounts for incubation with immature (GM-CSF- and IL-4-treated) and mature (GM-CSF-, IL-4-, and TNF-α-treated) moDCs. Twenty-four hours later, these moDCs were screened for a possible infection by fluorescence microscopy, and the proportion of infected cells was determined by FACS analysis. Interestingly, SeV-GFP particles were able to efficiently infect both immature as well as mature moDCs. Depending on the amount of incoming virus particles (MOI 1 or 2) the fraction of GFP-positive cells was higher in mature moDCs (56 or 82%) (Fig. 1, A and B). The increased number of GFP-positive cells using mature moDCs reflects a different level of susceptibility to SeV infection of immature and mature cells that cannot be explained by an increased level of particle endocytosis, because mature DCs are known to completely lose their endocytotic activity. To exclude an interference of LPS activation of moDCs and SeV infection, an additional experiment was performed by infecting LPS-stimulated moDCs (Fig. 1, E and F).

SeV infection impairs LPS-induced maturation of moDCs
Previous studies showed that viral infections may interfere with the phenotype or function of DCs. To determine the influence of SeV in this context, immature moDCs were infected with SeV-GFP (MOI 1), and FACS analysis of typical cell-surface markers was performed. As a result, some up-regulation of the surface markers CD80 and HLA-DR could be found, whereas a down-regulation of MHC class I molecules occurred (Fig. 2A). CD1a and CD86 revealed no relevant changes in their cell-surface expression. It is well known that the phenotype of DCs depends on the maturation status of these cells. A powerful mediator of DC maturation is bacterial LPS, which induces a rapid change of molecules displayed at the DC surface. Therefore, we next analyzed whether a SeV infection interferes with the capacity of moDCs to adequately respond to LPS. For this purpose, moDCs were first infected with SeV-GFP (MOI 1). Twenty-four hours later, moDC cultures were incubated with LPS for an additional 24-h time period, and surface markers were subsequently analyzed by FACS analysis. In contrast to noninfected moDCs (Fig. 2B, right column), SeV-infected moDCs did not show a characteristic LPS-induced maturation pattern (Fig. 2B, left column), represented by an inadequate increase in the expression of CD80, HLA-DR, CD40, CD83, CD86, and MHC class I molecules. These observations led to the assumption that SeV infections of DCs could severely impair the capacity of T cell stimulation.

SeV infection impairs the allostimulatory activity of mature moDCs
To this end, we tested moDC stimulatory activity to induce T cell proliferation in an allogeneic MLR. moDCs were infected with SeV-GFP and 24 h later were cocultured with allogeneic PBMCs for four additional days, followed by a 16-h pulse, after which the incorporation of BrdU was measured. Compared with uninfected moDCs, SeV-GFP infection severely impaired the T cell stimulatory capacity (Fig. 3). This functional impairment could be due to...
severe cellular alterations (as observed for the expression of MHC and costimulating molecules) generally induced in the course of SeV wild-type infections. This could be mediated by defined viral proteins interfering with a proper DC function (as it has been shown, for example, for HSV-1) (28).

Proinflammatory cytokine release pattern of virally infected moDCs
Recombinant viral particles, harboring deletions of single viral genes, can be used to investigate the contribution of the deleted genes to an observed biological phenomenon. In this context, single gene-deleted recombinant SeV particles can be used to test whether a specific SeV-encoded protein interferes with the function of infected moDCs. However, a direct comparison between different viral particles is only possible if these particles are able to infect moDCs with a similar efficacy. Thus, we first infected immature and mature moDCs with the following recombinant SeV particles (MOI 1 or MOI 2), analyzing the percentage of infected DCs by FACS analysis (Table I, DCs incubated with IL-4/GM-CSF or IL-4/GM-CSF/TNF-α): SeV missing the gene for the viral attachment protein hemagglutinin-neuraminidase HN (SeV-\text{HN-GFP}), the fusion protein F (SeV-\text{F-GFP}), or the matrix protein M (SeV-\text{M-GFP}). Interestingly, taking the SDs into account, all single gene-deleted particles demonstrated comparable infection rates to the SeV wild-type setting (SeV-GFP), even when using LPS-stimulated moDCs, thereby permitting a direct comparison between the different SeV particles used.

Subsequently, 48 h after infection of immature moDCs, we analyzed the cellular supernatant for the release of proinflammatory cytokines (IL-6, IL-10, IL-12, and TNF-α) known to play an important role in the Ag presentation and T cell stimulatory capacity of DCs. Interestingly, taking the SDs into account, all single gene-deleted particles demonstrated comparable infection rates to the SeV wild-type setting (SeV-GFP), even when using LPS-stimulated moDCs, thereby permitting a direct comparison between the different SeV particles used.

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**FIGURE 2.** Expression of surface molecules by SeV-infected moDCs. Immature moDCs were infected with SeV (wild-type) particles (MOI 1). A, Cell-surface expression of several molecules in uninfected (no infection) and SeV-infected (48 h) moDCs, analyzed by flow cytometry. B, Cell-surface expression in LPS-stimulated (for 24 h), uninfected, and SeV-infected dendritic cells, analyzed by flow cytometry. The percentage of cells per quadrant are listed in the top right corner for the corresponding positions of the quadrant. UL, Upper left; UR, upper right; LL, lower left; LR, lower right.
In the next set of experiments, we used the recombinant viral particles to determine the capacity of infected immature moDCs to respond to LPS stimulation. moDCs again were infected either with SeV-GFP or with one of the single gene-deleted recombinant viruses. Twenty-four hours after the initial infection, LPS was added to all cultures for an additional 24 h, and supernatants were analyzed for the release of cytokines (IL-6, IL-10, IL-12, and TNF-α). In contrast to the SeV wild-type situation (SeV-GFP), SeV-/H9004 F-GFP and SeV-/H9004 M-GFP showed an increased release of both IL-6 and IL-12 (Fig. 4 B). The amount of secreted IL-10 and TNF-α revealed only minor differences between the differentially infected moDC cultures (Fig. 4B). However, compared with uninfected control cells, the secretion of IL-10 was elevated in all infected DC cultures, which could contribute to a reduced MLR activity, as shown in Fig. 3.

Together, these observations implied a different reaction pattern of moDCs to the different SeV-derived particles used in this study. Therefore, an additional characterization of infected moDCs was performed.

Single gene-deleted SeV constructs revealed a reduced rate of apoptotic moDC cell death

A premature death of virally infected moDCs could be an additional reason of a reduced allostimulatory activity. We and others

**Table I. Infectivity of different SeV particles on DCs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>IL-4/GM-CSF</th>
<th>IL-4/GM-CSF/TNF-α</th>
<th>IL-4/GM-CSF/LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOI 1</td>
<td>MOI 2</td>
<td>MOI 1</td>
</tr>
<tr>
<td>SeV-GFP</td>
<td>47 ± 8</td>
<td>58 ± 5</td>
<td>56 ± 9</td>
</tr>
<tr>
<td>SeV-ΔM-GFP</td>
<td>38 ± 10</td>
<td>52 ± 7</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>SeV-ΔF-GFP</td>
<td>42 ± 7</td>
<td>61 ± 9</td>
<td>51 ± 9</td>
</tr>
<tr>
<td>SeV-ΔHN-GFP</td>
<td>39 ± 2</td>
<td>50 ± 10</td>
<td>48 ± 8</td>
</tr>
</tbody>
</table>

a Numbers represent the mean percentage of infected DCs and SD of three independent experiments.
b moDCs were generated from human PBMCs by incubation either with IL-4/GM-CSF (immature DCs) or with IL-4/GM-CSF/TNF-α (mature DCs).
c Immature moDCs were stimulated with LPS for 24 h before an infection with the different SeV particles.
reportedly that SeV wild type is able to induce apoptosis in viral host cells (27, 29, 30). Therefore, we next investigated whether infected moDCs underwent apoptotic cell death. Mature moDCs were either infected with SeV-GFP or one of the single gene-deleted viral recombinants (MOI 2) and analyzed apoptotic cell death induction by FACS analysis at two different time points (48 and 72 h after infection). Compared with uninfected control moDCs, SeV-GFP infection (wild-type situation) led to a profound increase in the rate of apoptotic cells (4 vs 13% after 48 h and 14 vs 25% after 72 h) (Fig. 5A). Interestingly, the single gene-deleted construct SeV-ΔM-GFP showed the lowest rate of apoptotic moDCs compared with all other virally infected cultures and differed only slightly from uninfected control cells (7 vs 4% after 48 h and 14 vs 14% after 72 h) (Fig. 5A). These results clearly show that gene deletions in the viral genome alter the response and fate of moDCs to SeV infection.

SeV-induced impairment of allostimulatory activity can be overcome by using M-deficient recombinant viral particles

To further investigate the contribution of single SeV proteins for an impairment of moDC function, mature moDCs were infected with either one of the different recombinant constructs (MOI 2) before their use in an alloreactive MLR. Surprisingly, SeV-ΔM-GFP-infected moDCs induced a strong allostimulatory activity that nearly reached the activity of uninfected control moDCs (Fig. 6, A and B). Furthermore, the deletion of either the F or HN gene also led to a partial reconstitution, but not in the same manner as the deletion of M (Fig. 6, A and B). These results demonstrate that either one of the viral-encoded proteins, M, F, or HN, contributes to the functional impairment of virally infected moDCs; however, the deletion of the viral M gene led to an almost complete reconstitution of the T cell stimulatory ability of infected DCs.

One possible explanation for a differential allostimulatory activity of SeVV-GFP- and SeVV-ΔM-GFP-infected moDCs could be a differential regulation of cytokines such as IL-10 or IL-12. To explore a possible impact of these two cytokines in more detail, additional alloreactive MLR experiments were performed comparing the influence of moDC infection with full-length SeV-GFP (Fig. 6C, gray bars) with SeV-ΔM-GFP (Fig. 6C, black bars). First, modulation by IL-10 secretion was blocked by adding an Ab against the IL-10 receptor (aIL-10R), and in a second approach, IL-12 was added to the culture medium (IL-12). As a result, neither blocking the IL-10 receptor nor adding IL-12 to the culture

FIGURE 5. Apoptosis phenomena in virally infected moDCs. A, moDCs were infected with SeV-GFP or the recombinant SeV particles SeV-ΔM-GFP, SeV-ΔF-GFP, and SeV-ΔHN-GFP (MOI 2) and cultured for 48 h (B, □) or 72 h (B, □). After the culture period, the rate of apoptotic cells was evaluated by FACS analysis using propidium iodide staining (proportion of sub2n DNA). As an infection control, GFP expression was determined to be in the range between 72 and 79% for all used viral particles. The median values of triplicates and single SDs are shown. B, An example, representative FACS results are shown for uninfected DC control cells (DC 72 h control) and for SeV-GFP-infected DCs (DC 72 h SeV-GFP). The indicated sub2nDNA fraction of cells was considered to be apoptotic.

FIGURE 6. Regain of allostimulatory activity by using single gene-deleted SeV particles. A, Mature (TNF-α-incubated) moDCs were incubated with PBMCs (no infection; ■), incubated with medium alone (no PBMC; ○), or infected with one of the following viral particles (MOI 2) 24 h before the addition of PBMCs: SeV-GFP (△), SeV-ΔM-GFP (□), SeV-ΔF-GFP (▼), SeV-ΔHN-GFP (○). Successful infection was confirmed by GFP expression (80–85% of GFP-positive cells in FACS analysis). The mean values of OD from a colorimetric evaluation of triplicate experiments and single SDs are shown. B, T cell proliferation after incubation with 100,000 infected moDCs relative to incubation with the same number of uninfected moDCs. The results of triplicate experiments and single SD are shown. Statistical significant results compared with uninfected moDCs (no infection; * p < 0.05) are indicated. C, Missing influence of IL-10 and IL-12 on the allostimulatory activity of moDCs after SeV infection. Mature moDCs were infected with SeV-GFP (MOI 2), infected with SeV-ΔM-GFP (MOI 2), or incubated with medium alone (control; no infection) 24 h before the addition of PBMCs. Abs against the IL-10 receptor (aIL-10R) or recombinant IL-12 were added to the cell cultures. The mean values and single SDs of OD from a colorimetric evaluation of triplicate experiments with 100,000 DCs and 1.5 × 10^6 PBMCs are shown. Significant differences compared with uninfected control cells are indicated (*, p < 0.05).
medium was able to revert the observed impairment of the allostimulatory activity by full-length SeV-GFP. In contrast, as shown in Fig. 6A, infection with SeV-ΔM-GFP particles (Fig. 6C, black bars) did not lead to a significant impairment of the allostimulatory activity compared with uninfected control cells (Fig. 6C, white bars). In conclusion, a deregulation of a single cytokine such as IL-10 or IL-12 seems not to be responsible for the observed effects.

From a virological point of view, the deletion of a single viral gene theoretically could alter the transcription of other virally encoded genes substantially. Therefore, we next compared the transcription of the virally encoded GFP gene in SeV-GFP- and SeV-ΔM-GFP-infected moDCs by quantitative PCR analysis (Fig. 7). GFP-specific signals were related to tubulin as an internal control (dCT). Because the dCT value is inversely proportional to the number of specific mRNA within a given sample, high dCT values represent less copy numbers of GFP compared with tubulin; vice versa, low dCT values represent high detection levels of specific RNA species compared with tubulin. As a result, mRNA of GFP is slightly enhanced in SeV-ΔM-GFP-infected moDCs (Fig. 7, filled bar on the left) compared with SeV-GFP-infected cells (Fig. 7, open bar on the left), whereas the number of viral genomes is clearly higher in SeV-GFP-infected cells (Fig. 7, open bar on the right) compared with SeV-ΔM-GFP-infected moDCs (Fig. 7, filled bar on the right). In conclusion, although the influence of the different number of viral genomes within infected moDCs is unclear, we could not detect a substantial difference in the transcription rate of GFP within SeV-GFP- and SeV-ΔM-GFP-infected moDCs.

Recently, it was shown that members of the NF-κB family of transcription factors are important for the differentiation and function of DCs (31–34). Therefore, we analyzed SeV-infected moDCs as described previously (35, 36) but found that the nuclear localization of the RelB and c-Rel proteins was not affected in the infected DC populations (Fig. 8), suggesting that the observed impairment after SeV infection is not mediated by inhibition of NF-κB signaling pathways.

To further characterize the M protein-associated alterations in the more complex moDC phenotype, we finally compared the surface expression of CD1a, CD80, CD83, CD86, and MHC class I and II molecules between SeV-GFP and SeV-ΔM-GFP (MOI 2) and cultured for 48 h. For moDC activation, cells were incubated with LPS 24 h before preparing nuclear extracts. A, Nuclear localized RelB. B, c-Rel proteins were detected by SDS-PAGE and Western blot analysis. Ponceau S staining was conducted to confirm equal loading of the gel.

**FIGURE 7.** Comparison of GFP transcription in SeV-GFP- and SeV-ΔM-GFP-infected moDCs. Mature moDCs were infected either with SeV-GFP or SeV-ΔM-GFP (MOI 1). Total RNA was isolated 20 h after infection, and reverse transcription was performed using either a mRNA species-specific primer (GFP mRNA) or a primer specific for SeV genomes, complementary to the leader region of the negative-strand RNA genome (SeV genomes). Subsequently, quantitative PCR was performed using specific primers for GFP and in a second sample as a control for the housekeeping gene tubulin. GFP-specific signals were related to tubulin as an internal control (dCT). Because the dCT value is inversely proportional to the number of specific mRNA within a given sample, high dCT values represent less copy numbers of GFP compared with tubulin; vice versa, low dCT values represent high detection levels of specific RNA species compared with tubulin. Infection studies were conducted in triplicates and quantitative PCR conducted in quadruplicates; the mean and SDs of the quantitative PCR analysis are shown.

**FIGURE 8.** Nuclear localization pattern of RelB and c-Rel proteins in SeV-infected moDCs after LPS stimulation. Peripheral blood monocytes were cultured with GM-CSF and IL-4. Cells were infected with SeV-GFP or SeV-ΔM-GFP (MOI 2) and cultured for 48 h. For moDC activation, cells were incubated with LPS 24 h before preparing nuclear extracts. A, Nuclear localized RelB. B, c-Rel proteins were detected by SDS-PAGE and Western blot analysis. Ponceau S staining was conducted to confirm equal loading of the gel.

**Discussion**

The first barriers against pathogens such as viral particles are built up by epithelial surfaces that are known to be lined up with DCs constituting an important defense mechanism. Once a virus succeeds in the penetration of this barrier, detection or capture of these viruses by DCs leads to their activation, migration to lymphoid organs, and initiation of an appropriate immune response. A growing number of viruses have been found being capable of directly infecting DCs, leading to an interference of proper DC functions or even to a premature death of infected DCs (21). In this study, we could demonstrate a surprisingly effective infection of both immature as well as mature moDCs by the paramyxovirus SeV, leading to an infection rate of up to 58 and 82%, respectively, at MOI 2 as analyzed by reporter gene (GFP) expression in DCs. Most RNA viruses that are able to infect immature DCs have been shown to induce DC maturation, which can be monitored by a change in the expression of cell-surface molecules (21). For SeV, we could demonstrate a slight increase in CD80, HLA-DR, and HLA class I molecules (Table II), thus further explaining the results obtained in the MLR assay.

An infection of DCs has been reported for three other SeV-related members of the paramyxovirus family: parainfluenzavirus type 3 (38), respiratory syncytial virus (RSV) (39), and MeV (7, 8). Notably, all of these SeV-related viruses do induce a maturation of virally infected DCs, a premature apoptotic death of DCs, and an interference with the capacity of allogeneic T cell stimulation (7, 38–40). The underlying mechanisms for these phenomena in parainfluenzavirus type 3- or RSV-infected DCs have not yet been revealed in detail. For MeV, it has been postulated that apoptosis induction in DCs results from Fas-mediated signaling...
(41), and the functional impairments in the allostimulatory properties were associated with an inhibition of IL-12 production by infected DCs (42) or a TRAIL-mediated T cell depletion (43). Interestingly, the MeV surface glycoproteins hemagglutinin (H) and fusion (F) proteins, which both are synthesized and presented by infected DCs, might play a decisive role in the interference with DC functions by inducing fusion events between DCs and T cells or by inhibiting T cell proliferation through surface contact-dependent negative signaling (44).

Our results for SeV infection of moDCs now demonstrate that the viral glycoproteins HN and F as well as the matrix protein M are key components in the observed functional impairment of DCs. In a situation in which all of these viral proteins were present (SeV-GFP infection of DCs; wild-type situation), a strong impairment of the allostimulatory DC properties could be observed (Fig. 3), together with a decrease in the production of IL-12 (Fig. 4B). For MeV infection, fusion events between DCs themselves or between DCs and surrounding T cells triggered by the presence of the viral proteins H and F on the DC surface have been suggested as one major mechanism for the interference with proper DC functions (8). In contrast to these observations, in our setting, we could exclude the involvement of fusion events by choosing culture conditions that definitively excluded any activation of SeV F proteins due to a lack of trypsin supplementation in the culture media.

In several experiments, we could detect a weaker GFP signal in SeV-ΔM-GFP-infected moDCs compared with all other viral particles. The explanation of this phenomenon remains elusive because we could not detect a reduced infection rate (Table I) nor a reduced transcription efficacy of GFP within SeV-ΔM-GFP-infected moDCs (Fig. 7). This issue will have to be addressed in an additional study focusing on the contribution of the M protein to viral transcription and replication efficiencies in detail, because it has been investigated previously for the related parainfluenza virus rabies in a cell type different to moDCs (45).

Nevertheless, both surface proteins HN and F contributed to the reduced allostimulatory activity, which could be demonstrated by a partial functional improvement in MLR assays by using recombinant SeV that is either genetically devoid of HN (SeV-ΔHN-GFP) or F (SeV-ΔF-GFP). Until now, the adverse affect of F and HN on the stimulatory capacity of DCs has not been elucidated. For MeV, an alteration in the cytokine secretion profile of infected DCs, such as a decrease in IL-12 or an increase in IL-10 secretion, is thought to skew T cell responses (42). In our study, a decrease in IL-12 secretion was most prominent after infection of moDCs with SeV particles resembling the wild-type situation or being devoid of the viral HN protein. No difference could be found between either SeV-ΔF-GFP- or SeV-ΔM-GFP-infected DCs. Concerning IL-10 secretion, the amount of cytokine release into the supernatant was found to be similar for all different viral particles. Moreover, adding αIL-10R Abs or IL-12 to the culture medium, we could not detect a significant reversion of the full-length SeV-mediated impairment in the MLR activity. Together, we could not identify single cytokines playing a major role in this process.

Despite a proven influence of HN and/or F on the function of moDCs, the use of M-deficient recombinant SeV particles led to a nearly complete reversion of the allostimulatory capacity of infected DCs compared with uninfected control cells. Therefore, the contribution of HN or F to the functional impairment of SeV-infected DCs could be neglected in settings in which M proteins were not synthesized in DCs. M is considered to be the central organizer of SeV morphogenesis, linking the ribonucleoprotein complex with the surface glycoproteins. Viral assembly takes place on internal cytoplasmic membranes (46), and M is able to bind independently to either the F or the HN glycoprotein (47). Intracellularly, M directly interacts with tubulin and microtubules, and recent work even suggested that the interaction between M and tubulin might have an important role in the regulation of SeV transcription (48). Interestingly, the role of tubulin and the microtubule network in DCs has come into focus recently in the context of DC-activating pattern recognition receptors: TLR2 and TLR4 could not be located on the surface but inside DCs in colocalization with α-tubulin, indicating that microtubules serve as transport tracks for TLR vesicles (49). These results also suggested that an intracellular stimulation of TLR2 or TLR4 leads to an activation and maturation of DCs. Depolymerization of the microtubule network disrupted the intracellular expression of TLRs and profoundly inhibited IL-12 production (49). Taking these observations into account, it is tempting to speculate that an intracellular colocalization of M and TLRs could lead to a functional impairment of DCs that can be overcome by using M-deficient SeV particles. For SeV, an interaction with pattern recognition receptors has not yet been described, but for other paramyxoviruses, an interaction of the RSV fusion protein with TLR4 (50) or the MeV H protein with TLR2 (44) has already been found.

Finally, for future gene transfer applications of DCs (51), this work has several important implications. First, SeV vectors can be

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**Table II. Surface Ag expression of SeV-infected DCs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>CD1a</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
<th>HLA-DR</th>
<th>HLA-ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeV-GFP</td>
<td>190</td>
<td>70</td>
<td>56</td>
<td>413</td>
<td>1135</td>
<td>2090</td>
</tr>
<tr>
<td>SeV-ΔM-GFP</td>
<td>270</td>
<td>113</td>
<td>102</td>
<td>601</td>
<td>1425</td>
<td>2931</td>
</tr>
<tr>
<td>SeV-ΔF-GFP</td>
<td>271</td>
<td>129</td>
<td>152</td>
<td>573</td>
<td>891</td>
<td>1963</td>
</tr>
<tr>
<td>SeV-ΔHN-GFP</td>
<td>248</td>
<td>127</td>
<td>149</td>
<td>517</td>
<td>830</td>
<td>2320</td>
</tr>
</tbody>
</table>

*Numbers represent the mean fluorescent intensity of GFP-positive mature moDCs, infected with different SeV particles (MOI 1).*
used for an extraordinary efficient infection of human moDCs. Second, impairment of the allostimulatory capacity of DCs as induced by SeV wild-type infection can be efficiently overcome by using gene-deleted (preferentially M gene-deleted) recombinant SeV vectors. Third, the observation that M gene-deleted recombinant SeV particles did not lead to a profound alteration of DC functions in MLR assays qualifies SeV-ΔM vectors as new, interesting candidates for genetic DC manipulation. In addition to the observations made in this study, SeV-ΔM vectors exhibit the benefit that no viral particles can be released from infected cells (52), which is an important safety issue for possible clinical applications of this vector system in the future.

Acknowledgments
We thank Silvia Stephan and Brunhilde Schuster for excellent technical assistance.

Disclosures
The authors have no financial conflict of interest.

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