Measles Virus (MV) Nucleoprotein Binds to a Novel Cell Surface Receptor Distinct from FcyRII via Its C-Terminal Domain: Role in MV-Induced Immunosuppression

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Morbilliviruses cause severe diseases in humans and animals (3, 27, 63). Most of these infections are associated with the development of a transient but strong immunosuppression. MV nucleoprotein (MV-N) has been reported to induce both cellular and humoral immune responses and paradoxically to account for immunosuppression. Thus far, this latter activity has been attributed to MV-N binding to human and murine FcyRII. Here, we show that apoptosis of MV-infected human thymic epithelial cells (TEC) allows the release of MV-N in the extracellular compartment. This extracellular N is then able to bind either to MV-infected or uninfected TEC. We show that recombinant MV-N specifically binds to a membrane protein receptor, different from FcyRII, highly expressed on the cell surface of TEC. This new receptor is referred to as nucleoprotein receptor (NR). In addition, different Ns from other MV-related morbilliviruses can also bind to FcyRII and/or NR. We show that the region of MV-N responsible for binding to NR maps to the C-terminal fragment (NTAIL). Binding of MV-N to NR on TEC triggers sustained calcium influx and inhibits spontaneous cell proliferation by arresting cells in the G0 and G1 phases of the cell cycle. Finally, MV-N binds to both constitutively expressed NR on a large spectrum of cells from different species and to human activated T cells, leading to suppression of their proliferation. These results provide evidence that MV-N, after release in the extracellular compartment, binds to NR and thereby plays a role in MV-induced immunosuppression.

During acute measles virus (MV) infection, an efficient immune response occurs, followed by a transient but profound immunosuppression. MV nucleoprotein (MV-N) has been reported to induce both cellular and humoral immune responses and paradoxically to account for immunosuppression. Thus far, this latter activity has been attributed to MV-N binding to human and murine FcyRII. Here, we show that apoptosis of MV-infected human thymic epithelial cells (TEC) allows the release of MV-N in the extracellular compartment. This extracellular N is then able to bind either to MV-infected or uninfected TEC. We show that recombinant MV-N specifically binds to a membrane protein receptor, different from FcyRII, highly expressed on the cell surface of TEC. This new receptor is referred to as nucleoprotein receptor (NR). In addition, different Ns from other MV-related morbilliviruses can also bind to FcyRII and/or NR. We show that the region of MV-N responsible for binding to NR maps to the C-terminal fragment (NTAIL). Binding of MV-N to NR on TEC triggers sustained calcium influx and inhibits spontaneous cell proliferation by arresting cells in the G0 and G1 phases of the cell cycle. Finally, MV-N binds to both constitutively expressed NR on a large spectrum of cells from different species and to human activated T cells, leading to suppression of their proliferation. These results provide evidence that MV-N, after release in the extracellular compartment, binds to NR and thereby plays a role in MV-induced immunosuppression.

Morbilliviruses cause severe diseases in humans and animals (3, 27, 63). Most of these infections are associated with the development of a transient but strong immunosuppression that contributes to secondary infections and mortality (18, 27, 63). The Morbillivirus genus includes Measles virus (MV), Canine distemper virus (CDV), Rinderpest virus (RPV), Peste-des-petits ruminants virus (PPRV), Porcine distemper virus, Dolphin morbillivirus, and Porpoise morbillivirus. The nonsegmented, single-stranded, negative-sense RNA genome of morbilliviruses encodes six structural proteins. While hemagglutinin (H) and fusion (F) are external glycoproteins, the nucleoprotein (N), the phosphoprotein (P), the viral polymerase (L) and the matrix protein (M) are internal proteins. The P cistron also encodes the nonstructural V and C proteins, but their function is still to be clearly determined.

N packs the RNA genome to form a helical nucleocapsid (NC), which binds P and L proteins. The result of this association, the ribonucleoproteic complex, constitutes the template for the transcription and replication of the viral genome. However, N has also the ability to self-assemble onto cellular RNA in the absence of viral RNA and viral proteins (4, 25).

MV-N consists of two regions: an N-terminal moiety, NCORE, and a C-terminal moiety, NTAIL, which is intrinsically disordered and protrudes from the viral NC surface (21, 32). While NCORE (amino acids [aa] 1 to 400) has sequences required for N self-assembly and RNA binding (2, 9, 25, 31), NTAIL (aa 401 to 525) contains the regions responsible for the binding of both assembled and monomeric forms of N to P, as well as those involved in binding to the polymerase complex (P-L) (2, 19, 31). N interaction with intracellular partners relies also on NTAIL; in particular, the region of MV-N encompassing residues 415 to 523 binds to the interferon regulatory factor 3, leading to the activation of the alpha/beta interferon enhancer (54). A conserved, hydrophobic patch at the extreme C terminus of MV-N binds to the heat shock protein Hsp72, which modulates the level of viral RNA synthesis (66). Despite the fact that MV-N is not exposed at the surface of the virion and of infected cells, it interacts with an extracellular receptor (45).

During the acute phase of MV infection, both strong humoral and cellular anti-N immune responses have been described. At the onset of the rash, the humoral response occurs after B lymphocyte stimulation by accessible and native MV antigens. Notably, the most abundant and rapidly produced antibodies are N specific. The production of anti-N antibodies...
precedes that of anti-H, anti-F, and sometimes anti-M antibodies (17, 36). These observations suggest that MV-N is released from MV-infected cells in the extracellular compartment, thus becoming accessible to the B-cell receptor. Moreover, efficient helper CD4+ T and cytotoxic CD8+ T-cell responses against N are generated during the course of MV infection (23, 24, 59). MV-N induces, in the absence of other viral proteins, N-specific T-cell responses, a systemic anti-N antibody response, and protection against MV infection (12, 39). Thus, MV infection is associated with an effective N-specific response that leads, with other events, to viral clearance, recovery from disease in 2 weeks, and to a protective immune response against reinfection. Paradoxically, MV simultaneously induces an immunosuppression by indirect mechanisms occurring in uninfected cells. In addition to MV H and F, experimental evidences indicate that MV-N is also involved in MV-induced immunosuppression. Indeed, MV-N binds to human and murine cell surface Fc receptor for immunoglobulin G (IgG) of type II (FcγRII, CD32) (45) and suppresses the inflammatory immune response in vivo in a murine delayed-type hypersensitivity model (34). This inhibition is associated with an inhibition of the antigen-specific CD8+ T-cell proliferation and a decrease of interleukin-12 (IL-12) production by murine dendritic cells (DC) (34). MV-N also inhibits in vitro IL-12 and antibody production in human CD40-activated DC and activated B lymphocytes, respectively (45, 55).

Here, we show that MV-N is released in the extracellular compartment after apoptosis of MV-infected human thymic epithelial cells (TEC). We report that MV-N can subsequently bind to cell surface via the interaction of NTAIL, with a specific receptor. We call this unidentified protein the nucleoprotein receptor (NR). We demonstrate that NR binds to other Morbillivirus-Ns but not that of the rabies virus (RV), a member of the Rabdovirus genus. Moreover, NR induces sustained calcium influx and inhibits both spontaneous and activated cell proliferation when triggered by MV-N. These results suggest that, in addition to MV-H and -F proteins, MV-N released in the extracellular compartment is involved in MV-induced suppression of cell proliferation.

MATERIALS AND METHODS

Cell lines and purification of primary cells. The P1.4D6 cortical TEC clone from human postnatal thymus was kindly provided by M. L. Toribio (13). The cell lines 3D1 (murine cortical TEC), HeLa (epithelial human carcinoma from cervix), MeWo (fibroblastic human melanoma from lymph node), NIH 3T3 (mouse fibroblasts from embryo), Jurkat (human acute T-cell leukemia), and Vero (monkey fibroblast from kidney) were obtained from the American Type Culture Collection (Manassas, Va.). The cell lines IIA1.6 (mouse B lymphoma, an FcγRII-negative variant of A2O/J cell line) and IIA1.6, expressing human FcγRIIb isoform (IIA1.6 Hu FcγRIIb1), were a gift from J. G. J. Van de Winkel (60). Cell lines were grown in RPMI 1640 or Dulbecco modified Eagle medium (Invitrogen, Inc., Grand Island, N.Y.) supplemented with 2 mM L-glutamine (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma) (for murine and monkey cell lines), and 5% to 10% fetal calf serum (FCS) (Biomedia).

Human T cells, monocytes, and DC were purified as previously described (14). Murine T and B cells were obtained from spleen. Murine macrophages were obtained after biogel dorsal injection, and DC were obtained from lymph node as described elsewhere (33). C57BL/6 mice were purchased from IFFA CREDO (L’Arbresle, France). Gene-targeted mice lacking FcγRII, FcγRI, and FcγRIIB (Fcer1g/Fggr2 double-knockout mice, model 585-MM) were obtained from Tac- onic.

Human T-cell activation. Freshly isolated T cells were activated at 105 cells/ml with a combination of 10 ng of phorbol myristate acetate (Sigma)/ml and 1 μg of ionomycin (Sigma)/ml in complete culture medium (RPMI 1640 + 10% fetal calf serum), as previously described (14). After 16 h of activation, T cells were washed and used for MV-N binding and functional assays.

Antibodies. The monoclonal antibodies (MAbs) used for the detection of Morbillivirus-N were biotinylated anti-MV-N C25 (IgG2b isotype), anti-MV C120 (IgG2b isotype) (16), anti-PPRV-N 38.4 (IgG1 isotype), anti-RV-N IVB-4 (IgG1 isotype), and anti-RP-V-N 33.4.4 (IgG1 isotype) (28). C. Kai (Tokyo, Japan) and M. Lafon (Paris, France) kindly provided anti-CD-N (clone 5) and recombinant anti-RV-N PVA3 (29), respectively. The 3F2 MAb was a 2.4G2, recognizing murine FcγRII (CD32) and FcγRII (CD16) (57), and the blocking mouse MAb KB61, recognizing all human FcγRIIIs (CD32), were kindly provided by D. Y. Mason (42). Phycocerythrin (PE) anti-human CD32 (Marseille, France), and anti-mouse CD16/CD32 (Pharmingen, San Diego, Calif.) MAbs were used. Mouse T and B cells, macrophages, and DC were visualized by using fluorescein isothiocyanate (FITC)-labeled anti-mouse CD3 (2C11, homemade), IgG [goat F(ab')2], fragment mouse IgG1, IgG-FITC (Immunotech), CD11b (Immunotech), and CD11c (Pharmingen) MAbs, respectively. Rat and mouse IgG1 and IgG2 isotypic controls (Immunotech) were used as isotypic controls of immunofluorescence.

CD25 MAb was adsorbed on polystyrene microparticles according to the manufacturer’s instructions (Polysciences). Briefly, 0.5 μl of bead suspension was washed in borate buffer prior to incubation with 400 μg of CD25 MAb overnight at room temperature. Beads were then extensively washed and stored at 4°C in 5% glycerol storage buffer. MAb adsorption on beads was then confirmed by flow cytometry with PE-conjugated goat anti-mouse IgG (Immunotech).

Cloning, production, and purification of Morbillivirus-N and RV-N. Viral MV-N was produced from Vero cells infected with MV Hallé strain at 0.01 PFU/cell as described previously (45). Recombinant MV-N was produced from confluent S9 cells infected with recombinant baculovirus AcNPVNP at 1 PFU/cell, as previously described (45). The PRPV gene (Nigeria 75/1 vaccine strain) (10) was subcloned into the plasmid pcAYMI under the control of the polyhedrin promoter and transferred into the baculovirus AcNPVNP by homologous recombination in transfected insect cells. Recombinant PPRV was produced in insect S9 cells (30). For the rinderpest N protein (RPV-N), the coding sequence of the N protein gene (RGK1 strain) was amplified by reverse transcription-PCR and cloned into the transfer vector pBacPAK6 (Clontech) downstream of the polyhedrin promoter. The final plasmid was used to generate the recombinant baculovirus RGK1-N by cotransfection of S9 cells with the Bm13 I-digested BacPAK6 viral DNA (Clontech). The gene encoding CDV-N from M13mp19 CDVPN (kindly provided by R. Buckland, Lyon, France) was cloned into the BamHI site of pFASTBac 1 (Invitrogen). Baculovirus CD-VN was prepared according to the instruction manual of Bac-To-Bac baculovirus expression system (Invitrogen). The recombinant baculovirus RV-VN was a gift from M. Lafon (Paris, France), and the corresponding cDNA was constructed as described previously (41). S9 cells were infected with RPV-N, PPRV-N, CDV-N, homoe, or RV-N. Recombinant baculoviruses at a multiplicity of infection of 1. Cells infected with RV or recombinant baculoviruses were lysed, and viral N or recombinant-N was purified on a preformed gradient of 50 to 30 to 25% (wt/vol) CsCl and 5 sucrose (wt/vol) as previously described (45).

Recombinant MV-N was also obtained from Escherichia coli as described elsewhere (25). The Ncore fragment, corresponding to aa 1 to 400 of MV-N, was obtained by limited proteolysis of purified N as described by Karlin et al. (25). The gene fragment of MV-N coding for Ncore (residues 401 to 525) was cloned into the pQE-32 vector (Qiagen), which allows the bacterial expression of a hexahistidine-tagged recombinant protein. Protein expression was performed as described previously (32). The protein was purified by immobilized metal affinity chromatography by using n-nitrotriacetic acid agarose (Qiagen). The identity of the purified product was confirmed by Western blotting with an anti-hexahistidine tag MAb from Qiagen (data not shown). Purified Morbillivirus-Ns were solubilized in phosphate-buffered saline (PBS) or in TNE buffer (20 mM Tris- HCl, 50 mM NaCl, 2 mM EDTA; pH 8) containing 20% glycerol for functional assays. N preparations were dialyzed against TNE buffer to remove functional assays. N preparations were dialyzed against TNE buffer to remove.

All virus stocks were free of mycoplasma.

MV infection and detection of N in cell-free supernatants by ELISA. TEC were seeded at 5 x 105 cells/cm2 for 24 h and then infected at a multiplicity of infection of 0.1 with Vero cell-derived MV strains for 2 h. MV strains were parental recombinant MV-Tag (derived from Edmonton strain) or MV-Tag devoid of V or C expression. After 48 h, the supernatants were harvested. Supernatants were harvested. Supernatants were harvested. Supernatants were harvested.
France. At different times postinfection, cell-free supernatants were collected for enzyme-linked immunosorbent assays (ELISAs) and for infectious MV particle production. The median 50% tissue culture infective dose (TCID50) was calculated as previously described (61). For ELISA assays, cell-free supernatants were UV inactivated (30 min at 254 nm) and twofold serially diluted. The N content was then measured by capture ELISA as described elsewhere (34). A reference MV-N curve was established by using serial dilutions of purified viral MV-N. MV-N purified on a sucrose gradient was used as an internal control to demonstrate that only free N and not virion-associated MV-N was measured in our ELISA conditions.

Cell viability and apoptosis analyses. DIOCS(3) (3,3′-dihexyloxycarboxyanine; Molecular Probes, Inc., Eugene, Ore.) and propidium iodide (PI) double staining was performed to detect the viability and mortality of TEC cultures by flow cytometry. Briefly, cells were incubated for 15 min at 37°C with 40 nM DIOCS(3) in culture medium to evaluate mitochondrial transmembrane potential (Δψm) (58). As Δψm decreases with cell commitment to apoptosis, DIOCS(3) stains living cells but not apoptotic cells. PI (0.5 μg/ml) was added before flow cytometry analysis by using a calibrat flow cytometer and CellQuest software (Becton Dickinson). Integrated fluorescence was measured, and data were collected from at least 20,000 events per sample.

TEC apoptosis was also assayed by using an FITC (Sigma) conjugate of the cell-permeable caspase inhibitor VAD-FMK (Promega, Madison, Wis.). Upon entry into the cells, it irreversibly binds to activated caspases and thus serves as a specific in situ marker for apoptosis. Attached and floating cells were pooled and incubated 20 min at room temperature in the presence of FITC-VAD-FMK at 5 μM. Cells were then washed twice, and FITC-VAD-FMK labeling was detected by flow cytometry. Integrated fluorescence was measured, and data were collected for at least 10,000 events.

Detection of Morbillivirus-N binding by flow cytometry. To determine Morbillivirus-N, MV-N, MV-N, or MV-N binding to cells, 5 × 105 cells were incubated for 1 h at 4°C with 100 μl of purified N (50 μg/ml) in the presence or in the absence of blocking anti-FcyRII MAb in staining buffer. After three washes, cells were incubated for 30 min at 4°C with biotinylated MAbs specific for MV-, PPV-, and RPV-N. Cells were then incubated with streptavidin-PE (Caltag Laboratories, Santa Cruz, Calif.) for 30 min at 4°C. For CDV-N binding assays, anti-CDV-N (clone 5) and PE-conjugated goat anti-mouse IgG were used. Labeled cells were analyzed by flow cytometry as previously described. The mean fluorescence intensity (MFI) value was determined to quantify N binding. For inhibitory binding assays, the MFI obtained in the absence of human or murine anti-FcyRII±III MAbs was considered 0% inhibition.

For dual immunofluorescence labeling, murine T cells, B cells, macrophages, and DC were first incubated with 2C11-FITC, anti-IgG-FITC, CD11b-FITC, and CD11c-FITC MAbs, respectively, and this step was followed by MV-N incubation in the presence or absence of the blocking 2.4G2 MAb, as described above. Pretreatment and cycloheximide treatment of TEC. Cells were resuspended at a concentration to 106 cells/ml in RPMI 1640 without FCS and incubated at either 37°C for 30 min with 1 mg of promase or 2 mg of tryps in/ml or at 37°C for 1 h with 100 μg of papain/ml (all enzymes were from Boehringer Mannheim). TEC were then washed three times and resuspended in RPMI 1640. The MV-N binding assay was then performed as described above.

To study the regeneration of MV-N-binding activity, pretreated TEC were resuspended in RPMI 1640 containing either 6% FCS alone or 10 μg of cycloheximide/ml, followed by incubation at 37°C for 4 h prior to the MV-N binding assay.

Competition of MV-N binding by flow cytometry. FITC was solubilized in 1 mg of sodium carbonate-bicarbonate buffer/ml according to the manufacturer’s instructions (Sigma). Recombinant MV-N was dialyzed prior to labeling against NaCl (0.15 M) for 24 h. MV-N was incubated with soluble FITC (35 μl of MV-N) for 2 h at 4°C in the dark. FITC-labeled MV-N (FITC-MV-N) was dialyzed against PBS for 72 h to remove FITC excess. Based on the assumption that the MV-N concentration was not affected by FITC labeling, increasing concentrations of FITC-MV-N were applied to TEC for binding assays. Cells were then analyzed by flow cytometry. For competition experiments, FITC-MV-N (25 μg/ml) was incubated with various amounts of unlabeled MV-N. The MFI obtained with FITC-MV-N alone was considered 100%.

RESULTS

Extracellular N release is associated with MV-induced apoptosis. Although N is commonly considered an intracellular protein, the abundant production of anti-N antibodies in the course of MV infection strongly suggests that N is released from infected cells, a possibility that we investigated. We used MV strains having different cytopathic activities (11) and a human TEC model in which apoptosis is dependent on MV replication (58). We first compared the ability of Tag/Edm, MV-V−, and MV-C− strains to propagate in TEC. As illustrated in Fig. 1A, large syncytia were observed in Tag/Edm and MV-V−-infected TEC at day 5 postinfection. Twenty to 30% of cells died of apoptosis (Fig. 1A), as assessed by caspase activation, blue trypan coloration, and Hoechst staining (data not shown). In contrast, MV-C− infection induced a moderate cytopathic effect, with small syncytia and a very low level of apoptosis (9.6%, Fig. 1A). Moreover, we observed efficient production of viral particles by TEC infected with Tag/Edm and MV-V− compared to TEC infected with MV-C− (Fig. 1A). We then analyzed the ability of these strains to release N in the cell-free supernatant by ELISA. As shown in Fig. 1B, high amounts of N were detected in cell-free culture supernatants of Tag/Edm and MV-V−-infected TEC, reaching up to 12 μg/ml on day 5. In contrast, MV-C− was not detected in the cell-free culture supernatants of MV-C−-infected TEC, sug-
suggesting that extracellular N release is dependent either on apoptosis of infected TEC or on the presence of MV-C protein.

Relationships between MV-induced apoptosis and N release are also supported by experiments performed with a FIP known to inhibit “cell-to-cell” virus spreading. As illustrated in Fig. 1A, FIP inhibited both syncytium formation and apoptosis in MV-infected TEC (93 to 100% of inhibition) but had no effect on virus particle production. Moreover, FIP inhibited MV-N release in culture supernatants on day 5 postinfection.

FIG. 1. Extracellular MV-N release after apoptosis of MV-infected cells. TEC were infected with recombinant MV Tag/Edm, MV-V\(^{+}\), or MV-C\(^{+}\) and then treated with or without the FIP Z-D-Phe-L-Phe-Gly-OH. (A) Inhibition of MV-induced cytopathic effect in TEC by FIP on day 5. Attached TEC were visualized by May-Grunwald–Giemsa staining, and syncytium formation was determined under light microscope. \(\pm\), \(+\), and \(+ + +\), relative intensities of the cytopathic effect as defined by the number and the size of the syncytia. The percentage of pooled attached and floating TEC was evaluated by measuring caspase activity by flow cytometry. The number of infectious virus particles produced (TCID\(_{50}\)/milliliter) is expressed as the mean ± the standard deviation (SD) of three different experiments. (B) Time course determination of extracellular N release in MV-infected TEC culture supernatants. Samples were prepared and assayed by ELISA as described in Materials and Methods. N concentrations were evaluated with known amounts of standard purified viral MV-N. (C) Inhibition of extracellular N release by FIP on day 5. The experiments were performed twice, and SD values were <10%.
(Fig. 1C), showing a correlation between apoptosis of MV-infected TEC and N release in the extracellular compartment. As a result, N becomes accessible to cell surface receptors. MV-N binds to cells expressing or lacking FcγRII. To determine whether released N is capable of binding to the cell surface, living MV-infected TEC were assessed for MV-N cell surface detection by using a biotinylated anti-N MAb (Cl25) and streptavidin-PE. As shown in Fig. 2A, MV-N on the TEC cell surface was detected from 3 to 7 days postinfection, indicating that, after N release, MV-N binds to neighboring cells. To further study the ability of N to bind to TEC, we used both recombinant MV-N purified from insect cells and viral MV-N purified from MV-infected Vero cells. Murine IIA1.6 and human TEC cell lines, both negative for the expression of FcγRII (CD32), as well as a murine IIA1.6 cell line expressing the human FcγRIIb1 (IIA1.6 Hu FcγRIIb1), were used (Fig. 2B). TEC also failed to express both FcγRI (CD64) and FcγRIII (CD16) (data not shown). All of these cell lines, expressing or not expressing FcγRII, efficiently bound recombinant and viral MV-N (Fig. 2C and data not shown). MV-N binding to IIA1.6 and TEC was not affected by blocking anti-FcγRII MAb, indicating that MV-N binding does not occur through FcγRII (Fig. 2C). In contrast, the addition of blocking anti-FcγRII MAb partially inhibited (50 to 65% of inhibition) MV-N bind-
ing to the cell surface of IIA1.6 Hu FcγRIIb1 (Fig. 2C). Altogether, these results show that MV-N binds both to cells expressing or lacking FcγRII, suggesting the existence of a novel cell surface receptor for MV-N.

MV-N specifically binds to a novel cell surface protein receptor. We next investigated the biochemical characteristics of the MV-N receptor expressed on TEC. Cells were incubated at 37°C for 30 min with pronase prior to MV-N-binding assays.

FIG. 3. Specific binding of MV-N to a protein receptor distinct from FcγRII. (A) Requirement of protein synthesis for recovery of MV-N-binding activity after protease treatment. TEC were untreated (left panel) or treated with pronase (middle panel) before MV-N binding. Pronase-treated TEC were incubated for 4 h at 37°C in the absence (gray histogram) or in the presence of cycloheximide (thin line), prior to MV-N binding (right panel). As a negative control, cells were incubated with specific anti-MV-N (Cl25) MAAb and streptavidin-PE in the absence of MV-N (dotted line). (B) Dose-dependent binding of FITC-labeled MV-N. TEC were incubated 1 h at 4°C with various amounts of FITC-MV-N, followed by extensive washes prior to flow cytometry analysis. (C) Competition between unlabeled MV-N and FITC-MV-N. Cells were incubated with FITC-MV-N (2.5 μg/well, corresponding to 50% of binding) and increasing amounts of unlabeled MV-N. Percentages of labeled MV-N binding were reported and calculated assuming that the MFI observed with FITC-MV-N alone is 100%. The results are representative from one of three independent experiments.
As shown in Fig. 3A, pronase treatment of TEC resulted in the inhibition of MV-N binding: 90% of untreated cells were competent for MV-N binding (left panel), whereas only 25 to 30% bound MV-N after pronase treatment (middle panel). To analyze the regeneration of MV-N binding, pronase-treated TEC were cultured for 4 h at 37°C in the presence or absence of cycloheximide prior to MV-N binding assays (Fig. 3A, right panel). When pronase-treated TEC were incubated for 4 h in medium alone, up to 80% of MV-N binding was recovered (gray histogram). In contrast, the proportion of MV-N binding remained between 10 to 18% when cycloheximide was added to the medium (thin line). Similar results were obtained with papain or trypsin treatment (data not shown). These results demonstrate that protein synthesis is required for MV-N binding to occur on the cell surface of TEC.

To further demonstrate the specificity of MV-N binding to TEC, we used recombinant MV-N from insect cells labeled with fluorescein (FITC-MV-N). As illustrated in Fig. 3B, FITC-MV-N binding is saturable. Furthermore, competition experiments indicated that a 10-fold excess of unlabeled MV-N is required to inhibit FITC-MV-N binding by 70% (Fig. 3C). Altogether, these results support the conclusion that binding of MV-N to TEC is mediated by a specific protein receptor: the NR.

Both Morbillivirus-N and N\text{TAIL} but not N\text{CORE} bind to NR. Since the amino acid sequence of N is well conserved within the Morbillivirus genus (10), we hypothesized that PPRV-N, RV-N, and CDV-N may share common binding properties with MV-N. Like MV-N, purified recombinant PPRV-N and CDV-N bound both to cells expressing or lacking FcγRII (Fig. 4A). Conversely, binding of RV-N did not involve FcγRII, as indicated by the lack of binding to Hu FcγRIIb1 (Fig. 4A). To exclude the possibility that this binding activity may be due to the self-polimerization property of Morbillivirus-N, we used the unrelated NC from RV, a virus belonging to the Rhabdoviridae family. Like MV-N, the unrelated NC RV-N possesses the ability to self-assemble to form NC (41). No RV-N binding was detected on TEC expressing NR (data not shown), suggesting that NR is not a receptor for NC structures but binds specifically to Morbillivirus-N.

Since the N\text{TAIL} domain of Morbillivirus-N protrudes from the surface of viral NC (21), we hypothesized that binding of MV-N to NR may be mediated by N\text{TAIL}. To test this possibility, we compared the ability of NR to bind to N\text{TAIL} and N\text{CORE} purified from bacteria. As shown in Fig. 4B (top, left panel), MV-N was partially degraded, as already reported in the case of MV-N purified from virions (15). Limited proteolysis of bacterially purified MV-N in the presence of trypsin resulted in the obtention of a 43-kDa fragment, which corresponds to N\text{CORE} (Fig. 4B). No fragment corresponding to the remaining 15 to 17 kDa (N\text{TAIL}) was detected, indicating that it is hypersensitive to proteolysis and entirely degraded (25). The bacterially purified N\text{TAIL} domain is also shown in Fig. 4B (top, right panel).

Two anti-MV-N MAbs, CI120 (recognizing aa 133 to 140) and CI25 (recognizing aa 466 to 474), were used to specifically detect N\text{CORE} and N\text{TAIL}, respectively (Fig. 4B, bottom). In contrast to MV-N, no binding of N\text{CORE} to NR is detected with anti-N CI120 MAb, even with N\text{CORE} amounts as high as 30 μg/well (Fig. 4C, left panel, and data not shown). Using the anti-N CI25 MAb, but not with anti-N CI120 MAb, we observed binding of N\text{TAIL} to NR (Fig. 4C, right panel, and data not shown). As for MV-N, binding of N\text{TAIL} to NR was not blocked by anti-FcγRII MAb (data not shown). In addition, protein synthesis was also required for N\text{TAIL}-binding activity on the cell surface of TEC (data not shown). To further demonstrate the specific binding activity of N\text{TAIL} to TEC, we performed competition experiments by coincubating 2.5 μg of purified MV-N/well with increasing amounts of N\text{TAIL}. As shown in Fig. 4D, N\text{TAIL} significantly inhibited the binding of MV-N (55 to 70%) in a dose-dependent manner, whereas N\text{CORE} fails to do so. Altogether, these results demonstrate that the region responsible for specific binding to NR is located within the N\text{TAIL} domain of MV-N.

Recombinant MV-N is internalized in TEC. We then analyzed the ability of MV-N to bind to the cell surface of TEC after incubation at 37 and 4°C. A strong decrease (20 to 70%) in the amount of MV-N detected on the cell surface was observed as a function of time when TEC were incubated at 37°C, whereas only a slight decrease was observed at 4°C (Fig. 5A, left panel). Similar results were obtained with N\text{TAIL} (Fig. 5A, right panel), thus ruling out the possibility that the self-assembled nature of MV-N is responsible for binding to NR. The observed drop in the amount of MV-N on the cell surface suggested that it may be internalized. This hypothesis was confirmed by confocal microscopy analyses (Fig. 5B). MV-N was exclusively present at the plasma membrane level when incubated at 4°C (upper panels). However, no uniform fluorescence was observed, suggesting cell surface MV-N/NR clustering. After 1 h at 37°C, cellular redistribution of MV-N was observed: in particular, through a strong decrease of the fluorescence at the plasma membrane, with the concomitant accumulation in vesicle-like structures within the cytoplasm (lower panels). These results suggest that internalization may occur either by fluid-phase pinocytosis or receptor-mediated endocytosis. MV-N internalization is not toxic for TEC, as indicated by the fact that no apoptosis, as judged by Hoechst staining, was observed after 1 h at both 4 and 37°C (data not shown).

Recombinant MV-N binding to NR on TEC induces cell cycle growth arrest rather than apoptosis. We investigated whether MV-N could affect TEC biology through NR engagement. For this purpose, we studied Ca\textsuperscript{2+} mobilization. A transient intracellular free Ca\textsuperscript{2+} increase in response to the ionophore ionomycin was observed (Fig. 6A). In the presence of MV-N, changes in Ca\textsuperscript{2+} concentration were observed. First, MV-N alone induced a small, but significant, increase in the intracellular free Ca\textsuperscript{2+} basal level. Second, costimulation with ionomycin and MV-N elicited a sustained Ca\textsuperscript{2+} signal.

Since changes in the Ca\textsuperscript{2+} concentration affect the balance between proliferation and differentiation in epithelial cells (5, 6, 37, 56, 65), we therefore examined whether MV-N binding modulates TEC proliferation. As illustrated in Fig. 6B, MV-N, purified from insect cells (not shown) and bacteria, inhibited spontaneous TEC proliferation in a dose-dependent manner (18 to 95%, left panel). Moreover, N\text{TAIL} (right panel), but not N\text{CORE} (middle panel), also inhibited TEC proliferation by 10 to 95%, indicating that the inhibition of cell proliferation is NC independent.

We next analyzed the cell cycle distribution of MV-N-treated TEC. As illustrated in Fig. 6C, we observed a significant decrease (81% inhibition) in the percentage of MV-N-
FIG. 4. *Morbillivirus*-N and N\textsubscript{T}AIL binding to cell surface receptor(s). (A) Binding of *Morbillivirus*-N to different cell lines expressing or not expressing FcyRII. The extent of binding has been evaluated as described in Materials and Methods. The MFI is indicated as follows: -, no binding; +, ++, and ++++, MFI values of ca. 10, between 10 and 100, and >100, respectively. The results are representative of three to six independent experiments. (B) At the top of the panel is shown MV-N domain purification from bacteria. Purified proteins were separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. M, molecular mass markers. At the bottom of the panel is shown MV-N domain organization. MV-N (aa 1 to 525) is divided into two domains: N\textsubscript{CORE} (aa 1 to 400) and N\textsubscript{T}AIL (aa 401 to 525). The epitopes recognized by the anti-MV-N C1120 and C125 MAbs are indicated. N-N and N-RNA binding sites are also shown. (C) Binding of MV N\textsubscript{T}AIL, but not N\textsubscript{CORE}, to NR. TEC were incubated with 5 \(\mu\)g of purified MV-tagged N (thin line) prior to flow cytometry analysis. Binding of N\textsubscript{CORE} and N\textsubscript{T}AIL (5 \(\mu\)g, heavy line) was detected by using biotinylated anti-MV-N C1120 (left panel) and C125 (right panel), respectively, and then revealed with streptavidin-PE. As a negative control, neither N nor N domains were added, and the cell lines were incubated in the presence of specific biotinylated anti-N MAb and streptavidin-PE (dotted line). The results are representative from one to three independent experiments. (D) Specific N\textsubscript{T}AIL binding to NR. Competition binding experiments were performed by incubating MV-N (2.5 \(\mu\)g, corresponding to 50% of binding) with increasing amounts of either N\textsubscript{T}AIL or N\textsubscript{CORE}. Bacterially purified MV-N binding was revealed by biotinylated anti-MV-N C1120 or C125 MAbs, depending upon whether N\textsubscript{T}AIL or N\textsubscript{CORE} was used as a competitor, respectively. The percentage of MV-N binding inhibition was calculated assuming that the MFI observed with MV-N alone represents 0% of inhibition. The results are representative from one to three independent experiments.
treated TEC in the S phase of the cell cycle by day 2 compared to untreated cells (left panel). Moreover, an accumulation in the G0/G1 of the cell cycle (70%) was subsequently observed after MV-N treatment (right panel). Finally, neither MV-N-, NTAIL-, nor NCORE-treated TEC undergo apoptosis, as shown by DIOC6/PI or Hoechst staining (Fig. 6D and data not shown). Altogether, these results demonstrate that MV-N is not toxic but specifically induces a cell cycle arrest rather than apoptosis. Moreover, these results indicate that the NTAIL domain is responsible for these biological effects.

Recombinant MV-N binds to NR on a large number of cell types and species. We then focused on the cellular distribution of putative MV-N receptor(s) different from FcγRII. We used human, murine, and monkey cell lines, as well as freshly isolated human and murine cells (from wild-type [wt] mice or mice lacking FcγRII/III). As illustrated in Table 1, binding of MV-N to human, murine, or monkey epithelial cells and fibroblasts was observed. Moreover, no inhibition of MV-N binding is obtained after treatment with blocking anti-FcγRII/III MAb, indicating that MV-N binds to NR. MV-N binding to human or

FIG. 5. MV-N constitutive internalization in TEC. (A) Downregulation of MV-N binding in TEC. MV-N binding to TEC was measured at either 4 or 37°C flow cytometry. Binding of recombinant MV-N (left panel) and NTAIL (right panel) was revealed by using anti-MV-N (Cl25) and streptavidin-PE. The results are representative of three independent experiments (SD values were <15%). (B) MV-N internalization at 37°C in vesicle-like structures within the cytoplasm. FITC-MV-N was used for confocal microscopy analysis. Single confocal sections show FITC fluorescence (left panel), transmission (middle panel), and overlap of both (right panel). Pictures are taken on TEC at either 4°C (upper panel) or 37°C (lower panel) after 1 h of incubation. Shown are representative results from one of three independent experiments. The white scale bar shown in the lower right panel corresponds to 5 μm.
wt murine monocytes, DC, and B cells was also observed. This binding activity was partially inhibited by anti-FcγRII/III MAb, suggesting that the binding of MV-N relies partly on FcγRII/III (Table 1). Binding of MV-N to NR on these cells was confirmed in FcγRII/III−/− mice (Table 1). NTAIL also bound to human NR on lymphoid and nonlymphoid cell surface (data not shown). Although no MV-N-binding activity was detected on red blood cells and both human and murine resting T cells,

FIG. 6. NR is functional upon MV-N binding. (A) MV-N sustains ionomycin-mediated Ca2+ mobilization in TEC. At the time indicated by the arrow, 2 ml of loaded TEC (2 × 106/ml) was stimulated with salt buffer, MV-N (40 μg/ml), and/or ionomycin (10−5 M). Cytosolic Ca2+ influx was then measured. (B) Effect of MV-N or NTAIL on spontaneous TEC proliferation. TEC were incubated with medium alone (open bar, control) and with various amounts of bacterially purified recombinant MV-N (solid bars), NCORE (dotted bars), or NTAIL (gray bars). Each value represents mean ± the SD of triplicate cultures. (C) Cell cycle analysis of TEC with 7AAD(DNA)/PY(RNA) staining. Two-dimensional analysis of DNA/RNA content is shown. The percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle are shown. TEC were untreated or treated for 2 days with 200 μg of MV-N/ml purified from insect cells. (D) Viability and apoptosis were analyzed by flow cytometry in untreated TEC and in MV-N-treated TEC as in panel C after DIOC6/PI double staining. Viable cells are DIOC6 positive and PI negative. In contrast, apoptotic cells have both a decrease of mitochondrial transmembrane potential and permeabilized membranes that render them DIOC6 negative and PI positive, respectively. The results are from one representative experiment out of three.
MV-N binds to a human Jurkat T-cell line independently of FcγRII expression (Table 1), suggesting that MV-N binds to NR on human activated T cells.

**Upon binding to NR on human activated T cells, MV-N inhibits cell proliferation.** To further demonstrate that NR is induced after T-cell activation, human T cells were freshly purified from the peripheral blood and then activated with phorbol myristate acetate plus ionomycin for 16 h. After extensive washes, MV-N was added, and activated T cells were assayed for FcR expression and MV-N-binding ability at different time intervals. Although no significant FcγRII expression was detected on the activated T-cell surface (Fig. 7A), MV-N binding was observed by as early as day 1 (Fig. 7B). This binding activity reached a maximum on day 2 and then slowly decreased on day 3, indicating that NR is readily induced after T-cell activation. Finally, we examined whether MV-N binding modulates T-cell proliferation. As shown in Fig. 7C, MV-N inhibited mitogen-induced T-cell proliferation in a dose-dependent manner. Finally, no apoptosis was observed under these conditions (data not shown), indicating that MV-N induces suppression of T-cell proliferation rather than apoptosis.

**DISCUSSION**

Combined mechanisms account for MV-induced immunosuppression (see reviews, references 38, 51, and 53). Among them, inhibition of cell proliferation of both MV-infected and uninfected cells has been reported. The latter activity suggested a role of MV proteins in such events. Thus far, H and F glycoproteins expressed at the surface of MV-infected cell, as well as binding of recombinant MV-N protein from insect cells to human and murine FcγRII/III, have been reported to suppress immune responses of uninfected cells (34, 45, 49). Although MV-N is considered as an internal protein located either in the extracellular compartment after apoptosis and/or secondary necrosis of
MV-infected human TEC. MV initially replicates in the respiratory tracts and then spreads to the local lymphoid tissue. This second viremia allows the spreading of the virus to other lymphoid organs, including the thymus, where a profound thymic injury, characterized by degenerative and/or necrotic lesions, occurs (8, 62). Human thymic stromal cells, particularly TEC, have been described as targets for MV during the acute phase of infection (35, 64). In a SCID-hu thymic implant model, MV infection of TEC cause apoptosis of uninfected thymocytes (1). These observations might be ascribed to apoptosis of TEC replicating MV in vitro (58). Interestingly, our findings clearly indicate that both MV replication and apoptosis of MV-infected TEC are required for extracellular release of high amounts of N. These observations are consistent with the fact

![Inhibition of human activated T-cell proliferation after MV-N binding to NR.](image)

**FIG. 7.** Inhibition of human activated T-cell proliferation after MV-N binding to NR. (A) FcγRII expression on human activated T cells was detected by using anti-CD32-PE at different times after stimulation. (B) MV-N binding was detected with specific biotinylated anti-MV-N (Cl25) and then revealed with streptavidin-PE prior to flow cytometry analysis. Cells were incubated with 5 μg of purified recombinant MV-N from insect cells. As a negative control, cells were incubated without MV-N in the presence of anti-MV-N (Cl25) and streptavidin-PE. The experiments were performed twice, and the SD values were <10%. (C) Effect of MV-N on human activated T-cell proliferation. Activated T cells were incubated with either medium alone or with various amounts of recombinant MV-N from insect cells for 24, 48, or 72 h. Each value represents the mean ± SD of triplicate cultures. The results are from one representative experiment out of three.
that MV-N is the major constituent of cytosolic MV proteins in infected cells and suggest that sufficient amounts of extracellular N are present in the thymus to be accessible to cell surface receptors. In agreement with this hypothesis, our results indicate that MV-N is detected on the cell surface of living MV-infected TEC. This suggests that N, once released in the extracellular compartment, is capable of interacting with cell surface receptor(s) on neighboring MV-infected and uninfected cells, affecting their biology.

Here, we report that viral and recombinant MV-N specifically interacts with a novel cell surface protein receptor on TEC, different from FcγRII, that we call NR. Genetic and biochemical studies of Morbillivirus-N have revealed that the hydrophobic N-terminal domain (N_{CORE}) is involved in RNA binding and NC assembly, whereas the flexible C-terminal domain (N_{TAIL}) is involved in binding to P (2, 7, 9, 25, 31, 32). For several reasons, we excluded that RNA and/or the self-assembled state of MV-N could be involved in binding of N to NR. MV-N binding to NR is specific and is not simply due to an interaction of NC at the cell surface, as indicated by the fact that the nonrelated NC from RV-N is not able to bind to NR. In addition, we show that bacterially expressed N_{TAIL}, which contains no RNA and has no self-assembling properties (32), is necessary and sufficient for binding to NR. Conversely, the N_{CORE} domain, which still forms NC-like structures (32), does not bind to NR and does not inhibit MV-N binding. Therefore, our results rule out the possibility that MV-N binding to NR can be mediated by N-associated RNA or by NC-like particles. Experimental evidence indicates that the N_{TAIL} domain of MV-N is accessible on the surface of NC-like structures (21). Moreover, recent data indicate that N_{TAIL} is an intrinsically unstructured monomer in solution (32). The presence of flexible regions exposed at the surface of the viral NC would ideally favor the interaction with different viral and cellular partners, including NR.

Constitutive expression of NR is not restricted to TEC, since it is also detected on lymphoid and nonlymphoid cells from human, monkey, and mouse sources. Even if we cannot conclude whether MV-N binds to the same receptor on these different cell types, one can speculate that NR might be ubiquitous and conserved among different species. According to this hypothesis, we found that four Morbillivirus-N proteins bind to receptors exposed at the cell surface of different cell lines from different species, which do not correspond to their natural hosts. Indeed, PPRV-N and CDV-N bind to both human FcγRIIs and human and murine NR, whereas RPV-N only binds to NR. These results suggest that most Morbillivirus-N proteins share conserved FcγRII and NR on their respective hosts and that these receptors may derive from a common ancestral receptor. In agreement with this hypothesis, mammalian FcγRIIs are conserved among species (22).

Notably, our data clearly indicate that MV-N/NR engagement on TEC leads to signal transduction by modulating Ca^{2+} influx and to the inhibition of spontaneous cell proliferation by inducing an arrest in the G_{0}/G_{1} phases of the cell cycle. We also show that MV-N or N_{TAIL} binding to uninfected TEC induces growth arrest independently of apoptosis. Growth arrest has also been noted upon interaction of MV-H and-F proteins with uninfected cells (20, 49, 52), strongly suggesting that MV viral proteins share the ability to inhibit cell prolifer-

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