Measles virus nucleoprotein induces cell-proliferation arrest and apoptosis through N\textsubscript{T}\text{TAIL}–NR and N\textsubscript{CORE}–Fc\textsubscript{R}IIIB1 interactions, respectively

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Measles virus (MV) nucleoprotein (N) is a cytosolic protein that is released into the extracellular compartment after apoptosis and/or secondary necrosis of MV-infected cells \textit{in vitro}. Thus, MV-N becomes accessible to inhibitory cell-surface receptors: Fc\textsubscript{R}IIIB and an uncharacterized nucleoprotein receptor (NR). MV-N is composed of two domains: N\textsubscript{CORE} (aa 1–400) and N\textsubscript{T}\text{TAIL} (aa 401–525). To assess the contribution of MV-N domains and of these two receptors in suppression of cell proliferation, a human melanoma HT144 cell line expressing (HT144IIB1) or lacking Fc\textsubscript{R}IIIB1 was used as a model. Specific and exclusive N\textsubscript{CORE}–Fc\textsubscript{R}IIIB1 and N\textsubscript{T}\text{TAIL}–NR interactions were shown. Moreover, N\textsubscript{T}\text{TAIL} binding to human NR predominantly led to suppression of cell proliferation by arresting cells in the G\textsubscript{0}/G\textsubscript{1} phases of the cell cycle, rather than to apoptosis. N\textsubscript{CORE} binding to HT144IIB1 cells primarily triggered caspase-3 activation, in contrast to HT144IIB1/IC\textsuperscript{–} cells lacking the Fc\textsubscript{R}IIIB1 intra-cytoplasmic tail, thus demonstrating the specific inhibitory effect of the N\textsubscript{CORE}–Fc\textsubscript{R}IIIB1 interaction. MV-N- and N\textsubscript{CORE}-mediated apoptosis through Fc\textsubscript{R}IIIB1 was inhibited by the pan-caspase inhibitor zVAD-FMK, indicating that apoptosis was dependent on caspase activation. By using N\textsubscript{T}\text{TAIL} deletion proteins, it was also shown that the region of N\textsubscript{T}\text{TAIL} responsible for binding to human NR and for cell growth arrest maps to one of the three conserved boxes (Box1, aa 401–420) found in N of \textit{Morbillivirus}. This work unveils novel mechanisms by which distinct domains of MV-N may display different immunosuppressive activities, thus contributing to our comprehension of the immunosuppressive state associated with MV infection. Finally, MV-N domains may be good tools to target tumour cell proliferation and/or apoptosis.

INTRODUCTION

Measles virus (MV), belonging to the genus \textit{Morbillivirus} of the family \textit{Paramyxoviridae}, is an enveloped virus with a non-segmented single-stranded negative-sense RNA genome. It encodes two envelope glycoproteins, the haemagglutinin (H) and the fusion (F) proteins required for MV cell entry, a matrix protein (M)\textsuperscript{1} and three structural proteins involved in viral genome transcription and replication: the nucleoprotein (N), the phosphoprotein (P) and the large protein (L). The MV genome is encapsidated by N to form the helical nucleocapsid. The N–RNA complex binds to the viral polymerase complex, which consists of the P and L proteins (Curran & Kolakofsky, 1999). \textit{Morbillivirus} N consists of two domains: a highly conserved N-terminal moiety (N\textsubscript{CORE}, aa 1–400) and a poorly conserved C-terminal moiety (N\textsubscript{T}\text{TAIL}, aa 401–525) (Diallo \textit{et al.}, 1994). N\textsubscript{CORE} is globular and carries regions required for N self-assembly and RNA binding (Bankamp \textit{et al.}, 1996; Curran \textit{et al.}, 1993; Karlin \textit{et al.}, 2002; Liston \textit{et al.}, 1997). N\textsubscript{T}\text{TAIL} is an intrinsically disordered monomeric domain (Longhi \textit{et al.},...
MV infection induces both an efficient specific immune response and transient, but profound, immunosuppression contributing to secondary infections and mortality in humans (Beckford et al., 1985; Griffin, 1995; Miller, 1964). Virus clearance is ensured by specific immunity against MV proteins, particularly MV-N, which confers long-life protection against reinfection (Etchart et al., 2001; Olszewska et al., 2001) and includes N-specific T lymphocytes (Etchart et al., 2001; Ilonen et al., 1990; Jacobson et al., 1989; Olszewska et al., 2001; van Binnendijk et al., 1997). Although MV-N is a cytotoxic protein, the most abundant and rapidly produced antibodies during MV infection are N specific (Graves et al., 1984; Norrby & Gollmar, 1972). Thus, anti-N antibody synthesis indicates that MV-N is released into the extracellular compartment, where it binds to the B-cell receptor of antigen (BCR). Indeed, we have previously demonstrated that large amounts of MV-N are expressed on the surface of a large spectrum of normal cells, except human and murine resting T cells (Laine et al., 2003). NR detection on different cell species favours ubiquitous and conserved NR expression. Alternatively, MV-N may bind to a group of various receptors sharing similar binding properties. MV-N binding to human NR suppresses normal thymic epithelial and mitogen-activated T-cell proliferation by blocking cells in the G0/G1 phases of the cell cycle (Laine et al., 2003). Finally, in vitro antibody synthesis of activated human B lymphocytes expressing both FcRII and NR is dramatically reduced in the presence of MV-N (Ravanel et al., 1997).

In this work, we aimed to map the MV-N domains involved in the interaction with FcRII and NR and to determine the relative contribution of each receptor to the suppression of cell proliferation and to apoptosis. To this end, we used a melanoma cell line expressing or not expressing FcRII as a model. We showed that MV-N binds to human FcRII and NR through NCORe and NTail, respectively. While FcRII interaction with NCORe triggered apoptosis, the aa 401–420 region of MV-N acted predominantly by blocking cell proliferation in the G0/G1 phases of the cell cycle after binding to NR. Therefore, MV-N displays different suppressive activities depending on whether NCORe or NTail binds to its respective cell-surface receptor.

**METHODS**

**Antibodies.** The monoclonal antibodies (mAbs) used were: biotinylated anti-MV-N C25 (Giraudon & Wild, 1981), biotinylated anti-MV-N C120 (Giraudon & Wild, 1981) and anti-FLAG (clone M2; Sigma-Aldrich). The blocking mouse mAb KB61 recognizing all human FcRII isotypes was kindly provided by D. Y. Mason (Pullford et al., 1986). Phycoerythrin (PE)-conjugated anti-human FcRII (C1KM5 clone; Caltag Laboratories) mAb was also used. The secondary antibody used was PE-conjugated goat F(ab')2 fragment anti-mouse IgG (PE-GAM) from Immunotech. Streptavidin–PE (Av–PE; Caltag Laboratories) and mouse IgG1 and IgG2 isotypic controls (Immunotech) were also used.

**Cell lines.** The human melanoma cell line HT144, which does not express FcRII, was stably transfected with human FcRII cDNA (HT144IIB1) or with human FcRII cDNA lacking the intracytoplasmic tail (HT144IIB1/IIC+). The murine fibroblast L Orient cells transfected with human FcRII cDNA (L-CD32) were kindly provided by S. Lebecque (Schering-Plough, Dardilly, France). Cell lines were grown in RPMI 1640 (Invitrogen) supplemented with 2 mM l-glutamine (Invitrogen), 10 mM HEPES (Invitrogen), 40 μg gentamicin (Schering-Plough) ml-1 and 10% fetal calf serum (Biomedia).

**Production of MV-N, NCORe and NTail.** Recombinant MV-N (strain Edmonston B) was produced from Escherichia coli as previously described (Karlin et al., 2002). NCORe was obtained by limited proteolysis of purified N as described by Karlin et al. (2002), and NTail (strain Edmonston B) was purified as described elsewhere (Laine et al., 2003; Longhi et al., 2003).

**Construction of expression plasmids encoding NTail deletion proteins, and their expression and purification.** All NTail constructs were obtained by PCR using the plasmid pET21a[N (Karlin et al., 2002) encoding the MV-N protein (strain Edmonston B) as
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E. coli strain Rosetta (DE3) pLysS (Novagen) was used for the expression of NTAIL deletion constructs. Culture and induction conditions were as described by Longhi et al. (2003), except that chloramphenicol (17 µg ml⁻¹) was used instead of kanamycin.

Expression of tagged full-length NTAIL from the pQE32 vector was carried out as described by Longhi et al. (2003).

Purification of NTAIL proteins was carried out as described by Longhi et al. (2003). The proteins were purified by immobilized metal affinity chromatography (IMAC) using Chelating Sepharose Fast Flow Resin preloaded with Ni²⁺ ions (Amersham Pharmacia Biotech).

Protein concentrations were calculated as described by Longhi et al. (2003).

Detection of FCγRII. Direct immunofluorescence assays were performed in staining buffer (PBS containing 1 % BSA and 0-1 % sodium azide) as described by Laine et al. (2003). After labelling, cells were analysed (Cellquest software) by flow cytometry analysis using a Calibur flow cytometer (Becton Dickinson). Integrated fluorescence was measured and data were collected from at least 10,000 events.

Detection and competition of MV-N binding to cells, 5 x 10⁵ cells were incubated for 1 h at 4°C with 5 µg purified N (50 µg ml⁻¹) in staining buffer. As expected, the addition of KB61 mAb resulted in a strong reduction in binding of N-TAIL to L-CD32 cells (Fig. 1a). However, HT144 cells efficiently bound MV-N and N-TAIL and also FCγRII, strongly suggesting that FCγRII binding occurs through human NR. These results indicated that NR expression is not restricted to normal cells but that it is also expressed on malignant cell lines (Laine et al., 2003).

For cell-cycle analysis, cells were stained with 7-amino-actinomycin D (7AAD) and propidium iodide (PI) (Toba et al., 1995). Briefly, 20 µM 7AAD was incubated with 5 x 10⁵ cells and 1 µM PI was then added. Cells were analysed by flow cytometry. Data were collected from at least 20,000 events.

Apoptosis detection. Cells were stained with 7-amino-actinomycin D (7AAD) and propidium iodide (PI) (Toba et al., 1995). Briefly, 20 µM 7AAD was incubated with 5 x 10⁵ cells and 1 µM PI was then added. Cells were analysed by flow cytometry. Data were collected from at least 20,000 events.

RESULTS

MV-N is composed of NCORE and NTAIL domains (Fig. 1a). To investigate the MV-N domains responsible for binding to cell-surface receptors, we used either human melanoma HT144 cells expressing only NR or HT144IIB1 cells expressing both FCγRII and NR as a model (Fig. 1b). As expected, HT144IIB1 cells bound MV-N and NTAIL, but not NCORE, whatever amount was used (Fig. 1c, and data not shown), confirming that binding of NTAIL but not of NCORE occurs through human NR. These results indicated that NR expression is not restricted to normal cells but that it is also expressed on malignant cell lines (Laine et al., 2003). In addition, NTAIL binding to NR on HT144IIB1 cells did not affect FCγRII expression, as judged by the superimposition of the histogram compared with the controls (data not shown).

To confirm that NCORE interacts with FCγRII, we performed blocking experiments using anti-FCγRII mAb KB61. Biotinylated CI120 and CI25 mAbs were used to detect NCORE and NTAIL binding, respectively (Fig. 1a). Therefore, we used L-CD32 cells (Fig. 1b), since biotinylated CI120 mAb cross-reacted slightly with these cells. As illustrated in Fig. 1(d), L-CD32 cells efficiently bound MV-N, NTAIL and NCORE. As expected, the addition of KB61 mAb partially inhibited MV-N binding to L-CD32 cells (Fig. 1d). Interestingly, NCORE was responsible for binding to FCγRII, as judged by the complete inhibition of binding to L-CD32 cells in the presence of KB61 mAb (Fig. 1d). The fact that NCORE binding to L-CD32 cells in the presence of the high-affinity FCγRII inhibitory antibody KB61 was lower than that observed with the negative control (dashed line) may be accounted for by the cross-reactivity of CI120 mAb with L-CD32 cells. Thus, the addition of KB61 mAb would block NCORE binding as well as non-specific binding of CI120 mAb to L-CD32 cells. As expected, NTAIL binding to murine NR expressed on L-CD32 cells was not affected by KB61 mAb, as indicated by the superimposition of the...
histogram profiles obtained with and without anti-FcρRII mAb (Fig. 1d). Similarly, KB61 mAb did not affect Nτ binding to NR expressed on either HT144 or HT144IB1 cells (data not shown). Moreover, L Orient cells bound MV-N and Nτ but not NCORE (data not shown). Taken together, these results indicated the presence of NCORE–FcρRIIB1 and Nτ–NR interactions.

Both NCORE and Nτ domains of MV-N inhibit spontaneous cell proliferation

We next determined the contribution of the NCORE and Nτ domains to the suppression of cell proliferation through their interactions with FcρRIIB1 and NR, respectively. As shown in Fig. 2(a), MV-N and Nτ, but not
NCORE, inhibited up to 95% of HT144 cell proliferation. The apparent higher anti-proliferative activity of N TAIL compared with that of full-length MV-N could be accounted for by differences in the molar amounts of receptor engaged. As the molecular mass of N TAIL is approximately 15 kDa and that of N is approximately 60 kDa, a fourfold excess of N, compared with N TAIL, has to be added to yield the same molar amount and the same biological effect.

In contrast, NCORE, as MV-N and N TAIL, inhibited HT144IIB1 cell proliferation by up to 60% (Fig. 2b). Thus, the apparent discrepancy in the inhibitory effect between N TAIL and MV-N was more pronounced in HT144 cells than in HT144IIB1 cells (Fig. 2a versus b). This phenomenon was due to cumulative binding effects, i.e. the fact that MV-N binds to both FcRIIB1 and NR in HT144IIB1, while it binds only to NR in HT144 cells. In all cases, the observed inhibition was dose dependent (Fig. 2a and b), indicating that inhibition of cell proliferation specifically relies upon either NCORE–FcRIIB1 or NTAIL–NR interactions.

We next analysed the cell-cycle distribution of MV-N-, NCORE- or NTAIL-treated HT144 and HT144IIB1 cells by measuring the DNA/RNA contents. We observed a significant increase in the percentage of both cell types arrested in the G0/G1 phases (up to 35%) after MV-N treatment compared with untreated cells (Fig. 2c). Subsequently, a decrease in the percentage of MV-N-treated cells in both S and G2/M phases of the cell cycle by day 1 was observed (data not shown). Similar results were obtained with NTAIL but the difference was less marked (Fig. 2c, and data not shown). As
expected, N\textsubscript{CORE} induced a smaller increase (up to 21 %) in the percentage of G0/G1-arrested HT144IIB1 cells compared with MV-N. Conversely, no significant increase in the percentage of arrested HT144IIB1 cells was observed in the presence of N\textsubscript{CORE} (4 %; Fig. 2c). In conclusion, these results indicate that the N\textsubscript{CORE} and N\textsubscript{TAIL} domains of MV-N are responsible for human cell growth arrest through interaction with Fc\textsubscript{RIIB} and NR, respectively.

\textbf{N\textsubscript{CORE}–Fc\textsubscript{RIIB} interaction triggers cell apoptosis through caspase-3 activation}

HT144IIB1 cells treated with MV-N or N\textsubscript{CORE}, but not those treated with N\textsubscript{TAIL}, appeared dispersed and damaged compared with untreated cells, with some detaching and displaying apoptotic morphology (Fig. 3). When MV-N or N\textsubscript{TAIL} were added to HT144 cells, the monolayers also appeared dispersed, enlarged and displayed a round shape, although they remained attached to the culture dish, while the addition of N\textsubscript{CORE} did have this effect (Fig. 3). These results indicate that both MV-N and N\textsubscript{CORE}, but not N\textsubscript{TAIL}, efficiently trigger HT144IIB1 cell death through Fc\textsubscript{RIIB}.

We next investigated whether MV-N and N\textsubscript{CORE} mediated apoptosis via Fc\textsubscript{RIIB} by measuring caspase activation. Interestingly, HT144IIB1 cells underwent apoptosis when cultured with either MV-N (38·9 %) or N\textsubscript{CORE} (20·6 %), while N\textsubscript{TAIL} only slightly increased the level of apoptosis (5·9 %) compared with untreated cells (0·5 %; Fig. 3). Apoptosis of HT144IIB1 cells was also observed after CD32 cross-linking by Abs (AT10/CD32 plus rabbit anti-mouse, data not shown), suggesting that apoptosis resulted from clustering of Fc\textsubscript{RIIB}. A lower degree of apoptosis was observed in HT144 cells treated with MV-N and N\textsubscript{TAIL} (8·5 and 3·3 %; Fig. 3) compared with N\textsubscript{CORE} (1·6 %). These results strongly suggested that N\textsubscript{CORE}-induced Fc\textsubscript{RIIB} clustering triggered apoptosis, while NR-mediated apoptosis appeared to provide a minor contribution. In order to demonstrate that N\textsubscript{CORE}-induced apoptosis was strictly dependent on Fc\textsubscript{RIIB} clustering, we performed experiments on HT144 cells expressing Fc\textsubscript{RIIB} but lacking its intra-cytoplasmic tail (HT144IIB1/IC\textsuperscript{−}). In these cells, no signal can be transduced via Fc\textsubscript{RII} clustering. As expected, MV-N, N\textsubscript{CORE} and N\textsubscript{TAIL} bound to HT144IIB1/IC\textsuperscript{−} cells and the cells expressed Fc\textsubscript{RIIB} at a level similar to that of HT144IIB1 cells (Fig. 4a, and data not shown). Interestingly, neither MV-N nor N\textsubscript{CORE} triggered significant caspase-3 activation in HT144IIB1/IC\textsuperscript{−} cells (Fig. 4b), even though in some cases N\textsubscript{CORE}-binding detection was higher than that currently observed (data not shown). In contrast, MV-N and N\textsubscript{CORE} induced 24·7 and 14·2 % caspase-3 activation in HT144IIB1 cells, respectively (Fig. 4b). As expected, HT144IIB1 cells underwent apoptosis following the addition of either MV-N or N\textsubscript{CORE} as shown by nuclear condensation and fragmentation, in contrast to HT144IIB1/IC\textsuperscript{−} cells (Fig. 5a). Whichever cell line was used, N\textsubscript{TAIL} was not toxic, even with doses as high as 68 \(\mu\)g per well, as judged by pan-caspase activation, caspase-3 activation and Hoechst staining (Fig. 4b, 5a, and data not shown).

To demonstrate further that caspase activation was involved in MV-N- and N\textsubscript{CORE}-induced apoptosis through interaction with Fc\textsubscript{RIIB}, cells were incubated in the presence of the pan-caspase inhibitor zVAD-FMK and nuclear morphology was analysed at day 1. In HT144IIB1 cells treated with MV-N and N\textsubscript{CORE}, zVAD-FMK totally inhibited apoptosis, and the nuclei of untreated or N\textsubscript{TAIL}-treated cells remained intact (Fig. 5b). Taken together, these data demonstrated that both MV-N and N\textsubscript{CORE} specifically trigger apoptosis via binding to Fc\textsubscript{RIIB} in a caspase-3-dependent manner.

\textbf{Binding of conserved Box1 of Morbillivirus N\textsubscript{TAIL} to NR suppresses spontaneous cell proliferation}

In addition to the N\textsubscript{TAIL} domain of MV-N, three different N proteins derived from various members of the genus \textit{Morbillivirus} also bind to human NR (Laine \textit{et al.}, 2003). We thus hypothesized that the region(s) involved in NR binding may be located in one of the three conserved regions in the \textit{Morbillivirus} N\textsubscript{TAIL} (aa 401–420, 489–506 and 517–525) (Diallo \textit{et al.}, 1994). To test this possibility, we purified three N\textsubscript{TAIL} deletion proteins carrying different combinations of such boxes, N\textsubscript{TAIL\textsubscript{1}}, N\textsubscript{TAIL\textsubscript{2},3} and N\textsubscript{TAIL\textsubscript{3}} (Fig. 6a). These deletion proteins were all found in the soluble fraction of the bacterial lysate (Fig. 6b) and were purified by IMAC (Fig. 6b).

We then compared the ability of the N\textsubscript{TAIL\textsubscript{1}}, N\textsubscript{TAIL\textsubscript{2},3} and N\textsubscript{TAIL\textsubscript{3}} deletion proteins to bind to NR. Biotinylated C125 mAb was used to detect specific binding of these deletion proteins to HT144 cells. As shown in Fig. 6(c), both N\textsubscript{TAIL\textsubscript{3}} and N\textsubscript{TAIL\textsubscript{2},3} bound to NR, demonstrating that Box2 and Box3 are dispensable for NR binding. On the other hand, no detectable N\textsubscript{TAIL\textsubscript{1}} binding to NR was observed, even with amounts as high as 20 \(\mu\)g per well (data not shown), indicating that N\textsubscript{TAIL\textsubscript{1}} binding strictly requires Box1. Similar results were obtained when binding of deletion proteins was revealed with anti-FLAG mAb (data not shown). Binding of N\textsubscript{TAIL\textsubscript{3}} and N\textsubscript{TAIL\textsubscript{2},3}, but not N\textsubscript{TAIL\textsubscript{1}}, was also observed on both murine L and human thymic epithelial cell lines (data not shown). The intrinsic disorder of N\textsubscript{TAIL} rules out the possibility that the inability of N\textsubscript{TAIL\textsubscript{1}} to bind to cells may arise from structural changes induced by removal of Box1. We further performed competition experiments by co-incubating MV-N with increasing amounts of N\textsubscript{TAIL} deletion proteins. Binding of MV-N was then detected using biotinylated C120 mAb, specifically recognizing the N\textsubscript{CORE} domain. Both N\textsubscript{TAIL\textsubscript{1}} and N\textsubscript{TAIL\textsubscript{2},3} significantly inhibited MV-N binding (19–76 %) in a dose-dependent manner, whereas N\textsubscript{TAIL\textsubscript{1}} failed to do so (Fig. 6d). Collectively, these results support the conclusion that the region responsible for specific binding to NR is located within aa 401–420 of MV-N.

Finally, we investigated whether NR engagement by the
Fig. 3. Cellular morphology and apoptosis of HT144 and HT144IIB1 cells 24 h after treatment with MV-N, N\textsubscript{CORE} or N\textsubscript{TAIL}. HT144IIB cells were treated with 34 μg MV-N, 68 μg N\textsubscript{CORE} or 13 μg N\textsubscript{TAIL} per well in a 48-well plate. Control experiments were carried out using untreated cells. Photomicrographs were taken under a light microscope at the same original magnification (×400; Leica DM IRB). One representative experiment of three is shown. The percentage of attached and floating cells positive for activated pan-caspase was determined using FITC–VAD-FMK. Results correspond to the mean from at least three independent experiments (SD <15%).
deletion proteins affected HT144 cell proliferation. As illustrated in Fig. 6(e), NTAIL2 and NTAIL3 deletion proteins inhibited human cell proliferation in a dose-dependent manner, although to a lower extent than NTAIL. As expected, NTAIL1 was unable to inhibit HT144 cell proliferation, even at concentrations as high as 20 μg per well (Fig. 6e). Similar results were obtained using L and thymic epithelial cell lines (data not shown). Thus, the aa 401–420 region of MV-N is required for NR binding and, as a result, is responsible for cell proliferation inhibition.

**DISCUSSION**

The mechanisms responsible for MV-induced immunosuppression are multifactorial and involved MV-infected immune cells, apoptosis of both MV-infected and uninfected cells, interference with cytokine synthesis, induction of soluble inhibitory factors and abnormalities in both antigen-presenting cell and lymphocyte functions (Gerlier et al., 2005; Schneider-Schaulies & ter Meulen, 2002). However, the overall frequency of infected peripheral blood
lymphocytes is usually low during viraemia, supporting the notion that indirect mechanisms based on viral protein effects are mainly involved. Thus, the F–H complex binding to an as yet unknown receptor on uninfected T cells was shown to disrupt intracellular signalling pathways leading to the inhibition of lymphocyte proliferation (Avota et al., 2001). Likewise, MV-N exerts a suppressive activity after interaction with cell-surface receptors expressed on uninfected cells (Gerlier et al., 2005). In the present study, we have identified the MV-N domains involved in binding to human FcγRIIB1 and NR, and shown that MV-N mediates either suppression of cell proliferation or apoptosis, depending on the type of receptor involved.

Our results point to an exclusive interaction of N\textsubscript{CORE} with FcγRIIB1 and show that MV-N binds to FcγRIIB1 more efficiently than N\textsubscript{CORE}. This latter point is probably related to the increased rigidity of N\textsubscript{CORE} compared with N (Longhi et al., 2003), which possibly renders sequential and/or conformational epitopes less accessible to FcγRIIB1. FcγRIIB1 is not a receptor responsive to any nucleocapsid-like particles. While the N of MV, canine distemper virus (CDV) and peste-des-petits-ruminants virus (PPRV) bind to FcγRIIB1, rinderpest virus (RPV) N does not (Laine et al., 2003), thus supporting specific N\textsubscript{CORE} binding to FcγRIIB1. The distinctive behaviour of RPV-N may be due to its unique sequence properties within the putative region of interaction with FcγRIIB1. Although the amino acid sequence of N\textsubscript{CORE} is well conserved among Morbillivirus members (overall sequence similarity of 80%), the similarity drops to 40% for the aa 122–144 region (Diallo et al., 1994). This variable region, already described as an antigenic region (Giraudon et al., 1988), is low in hydrophobic clusters and may therefore form a loop exposed to the solvent (Karlin et al., 2003), possibly involved in binding to FcγRIIB1. The conserved serine 138, occurring in MV-N, CDV-N and PPRV-N, is replaced by a glycine residue in RPV-N (Diallo et al., 1994). This substitution may lead to a conformational change in RPV-N, thus resulting in a spatial conformation unsuitable for the proper interaction with FcγRIIB1.

Fig. 5. Effect of MV-N and N\textsubscript{CORE} on apoptosis of HT144IIB1 and HT144IIB1/IC\textsuperscript{-} cells. Cells were incubated in the absence (a) or in the presence (b) of the pan-caspase inhibitor zVAD-FMK (100 μM) for 1 h at 37 °C prior to incubation with 34 μg MV-N, 68 μg N\textsubscript{CORE} or 13 μg N\textsubscript{TAIL} per well in a 48-well plate. Control experiments were carried out using untreated cells. Apoptosis was measured by Hoechst staining after 24 h. Attached cells were observed using a Leica DM IRB microscope at a magnification of ×400. White arrows indicate nuclear condensation and fragmentation. Data are representative of one experiment of two or three independent experiments.
Fig. 6. Mapping of the NR binding site within NTAIL. (a) Schematic representation of NTAIL and NTAIL deletion proteins. NTAIL3, NTAIL2,3 and NTAIL1,2 mutants are devoid of Box3, Box2 plus Box3, and Box1, respectively. The three NTAIL deletion proteins contained a six-His tag fused to their N terminus (white boxes) and a C-terminal FLAG (black boxes). The α-helical molecular recognition element, i.e. the region involved in induced folding of NTAIL through binding to P (Bourhis et al., 2004), is indicated as an α-helix. (b) Purification profile of NTAIL deletion proteins from bacteria, analysed by 15% SDS-PAGE and stained with Coomassie brilliant blue. TF, Bacterial lysate (total fraction); SN, clarified supernatant (soluble fraction); IMAC, eluent from IMAC. Molecular mass markers (kDa) are shown. (c) Binding of NTAIL deletion proteins to NR expressed on HT144 cells. Cells were incubated with 5 μg purified NTAIL deletion proteins per well for 1 h at 4 °C (thin lines). Binding was detected with biotinylated (Biot) Cl25 mAb and revealed with Av–PE prior to flow cytometry analysis. As a negative control, cells were incubated with biotinylated Cl25 mAb and Av–PE in the absence of NTAIL deletion proteins (dashed lines). The results are representative of one of three independent experiments. (d) Competition between MV-N and NTAIL deletion proteins. Cells were incubated with MV-N (2 μg per well, corresponding to 50% binding) and increasing amounts of NTAIL deletion proteins. MV-N binding was then detected using mAb anti-N (Cl120) and Av–PE prior to flow cytometry analysis. Results are expressed as MFI of binding and are representative of one of three independent experiments. (e) Effect of NTAIL, NTAIL3, NTAIL2,3 and NTAIL1 on spontaneous HT144 cell proliferation at 2 days post-treatment. Thymidine incorporation was determined as described in Methods. One representative experiment of three is presented.
Interestingly, the RPV-N C-terminal domain is more antigenic than its N-terminal counterpart. Among the three highly immunogenic epitopes located at both the C and N terminus of RPV-N, only one (aa 520–525) is conserved in MV NTAIL (aa 519–523) (Buckland et al., 1989; Choi et al., 2003, 2004). These results suggest that the *Morbillivirus* N epitopes involved in immune activation are different from the regions involved in binding to cell-surface receptors and thus in immunosuppression.

We showed that NR binds Box1 (aa 401–420), which is well conserved among *Morbillivirus* members (Diallo et al., 1994). Moreover, Box1 is also well conserved among wild-type and vaccine MV strains, as judged by the comparison between the amino acid sequence of NTAIL from the Edmonston B, other vaccine strains and 48 wild-type strains. Four conservative substitutions (T402A, 1406T/V, A415S and L420I) occur individually in eight wild-type strains, and the conservative K405R substitution is observed in the majority of the wild-type strains (D. W. Kouomou & F. T. Wild, personal communication). This latter substitution is also observed in other *Morbillivirus* N capable of interacting with NR (Diallo et al., 1994; Laine et al., 2003). This analysis also revealed complete conservation of the sequence in the aa 407–414 region between Edmonston B and all of the wild-type strains. Thus, the high conservation of Box1 highlights the biological relevance of studies focused on the interaction between NR and NTAIL from Edmonston MV strain.

We also reported that cell-cycle arrest was mediated predominantly by the NTAIL–NR interaction in a human melanoma line, whereas apoptosis was mediated primarily by the NCORE–FcRIIB1 interaction in melanoma cells expressing FcRIIB1. FcRIIB1 aggregation by antibodies also results in inhibition of spontaneous and activated B-cell proliferation (Pearse et al., 1999). Few data are available concerning the role of N from single-stranded RNA viruses in the modulation of cell proliferation. The only available data concern the role of intracellular N from Borna disease virus and hepatitis C virus in blocking cell proliferation through interaction with cell-cycle regulators (Planz et al., 2003; Yao et al., 2003). As no inhibition of cell proliferation has been documented so far for intracellular MV-N, our results strongly suggest that the effect of NCORE–FcRIIB1 interaction on cell proliferation can be ascribed to B cells, while the NTAIL–NR interaction inhibits both B- and T-cell proliferation. Similarly, both wild-type and vaccine MV strains suppress both infected and uninfected B- and T-cell proliferation *in vitro* (Gerlier et al., 2005; Hahn et al., 2003; McChesney et al., 1987, 1988; Naniche et al., 1999; Schlender et al., 1996). There are several lines of evidence suggesting that cell growth arrest cannot be ascribed to infectious virus or conventional cytokines (Fujinami et al., 1998; Sanchez–Lanier et al., 1988; Sun et al., 1998). In addition to the role of H/F proteins from MV, RPV and PPRV (Heaney et al., 2002; Schlender et al., 1996), soluble anti-proliferative factors produced from dead MV-infected cells arrest uninfected B and T cells in the G0/G1 phases (Fujinami et al., 1998; Wang et al., 2003). As MV-N is released from apoptotic MV-infected cells (Gerlier et al., 2005), it would be interesting to determine whether MV-N is the factor responsible for growth arrest. In addition to the potent anti-proliferative effect of MV-N, aggregation of FcRIIB1 by NCORE triggers apoptosis via the intra-cytoplasmic tail of FcRIIB1. Previous data have already demonstrated that cross-linking of murine FcRIIB1 by a combination of antibodies is sufficient to induce apoptosis of B cells independent of BCR co-ligation (Pearse et al., 1999). However, the link between inhibition of cell proliferation and induction of apoptosis mediated by both MV-N and NCORE after binding to FcRIIB1 is difficult to establish, and we cannot exclude the possibility that the induction of apoptosis is the consequence of suppression of cell proliferation. To our knowledge, only hepatitis C virus CORE protein has been described to exert a pro-apoptotic effect in transfected cells (Realdon et al., 2004). Apoptosis of uninfected B and T lymphocytes by MV, CDV and PPRV has been described, and lymphopenia, primarily due to apoptosis of uninfected lymphocytes, seems to arise mainly from indirect effects of the viruses (Gerlier et al., 2005; Mondal et al., 2001; Okada et al., 2000; Schoesberger et al., 2005). Although RPV-induced apoptosis has been ascribed to direct cytopathogenic RPV infection (Stolte et al., 2002), we hypothesize that the four *Morbillivirus* members trigger cell growth arrest via NTAIL–NR interaction, with MV, CDV and PPRV being also involved in NCORE–FcRIIB1-induced apoptosis. The severe lymphopenia observed with measles patients does not occur with the vaccine strains (Okada et al., 2001). In the cotton rat model, wild-type MV strains induce a higher anti-proliferative effect than vaccine strains through H/F glycoproteins (Niewiesk et al., 1997; Pfeffer et al., 2003). However, high-titre measles vaccine administration to young infants increased mortality, thus suggesting that vaccine virus may mimic the immunosuppressive effects of wild-type MV (Moss & Polack, 2001). Thus, further studies are necessary to understand in more detail the contribution of MV-N to the mechanisms of immunosuppression following MV infection. Finally, the potential effect of MV-N on cell death via FcRIIB1, in addition to its potent and global anti-proliferation effect via NR, may represent a promising approach for the local treatment of cancer cells expressing FcRIIB1, as well as for cell-cycle manipulation of rapidly proliferating cells expressing NR.

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