Genetically engineered attenuated measles virus specifically infects and kills primary multiple myeloma cells

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The applicability of cytoreductive treatment of malignant diseases using recombinant viruses strongly depends on specific recognition of surface receptors to target exclusively neoplastic cells. A recently generated monoclonal antibody (mAb), Wue-1, specifically detects CD138+ multiple myeloma (MM) cells. In this study, a haemagglutinin (H) protein that was receptor-blinded (i.e. did not bind to CD46 and CD150) was genetically re-engineered by fusing it to a single-chain antibody fragment (scFv) derived from the Wue-1 mAb open reading frame (scFv-Wue), resulting in the recombinant retargeted measles virus (MV)-Wue. MV-Wue efficiently targeted and fully replicated in primary MM cells, reaching titres similar to those seen with non-retargeted viruses. In agreement with its altered receptor specificity, infection of target cells was no longer dependent on CD150 or CD46, but was restricted to cells that had been labelled with Wue-1 mAb. Importantly, infection with MV-Wue rapidly induced apoptosis in CD138+ malignant plasma cell targets. MV-Wue is the first fully retargeted MV using the restricted interaction between Wue-1 mAb and primary MM cells specifically to infect, replicate in and deplete malignant plasma cells.

INTRODUCTION

Measles virus (MV) is a negative-strand RNA virus, belonging to the genus Morbillivirus of the family Paramyxoviridae. The MV genome encodes six proteins, three of which participate in formation of the viral envelope, the matrix, haemagglutinin (H) and fusion (F) proteins. Both H and F are determinants for virus-to-host attachment and fusion. Upon infection, cells expressing F and H become fusogenic with neighbouring uninfected cells, leading to cell-to-cell fusion and virus propagation (Yanagi et al., 2006). The receptors of MV are well defined: CD46, which is a type I transmembrane protein that is expressed in four major isoforms, a member of the human complement regulatory proteins and an ubiquitous cellular receptor found on all nucleated cells (Dörg et al., 1993; Naniche et al., 1993), and CD150 or SLAM (signalling lymphocyte activation molecule), a 70 kDa membrane glycoprotein that is present on activated B cells, T cells and monocytes (Tatsuo et al., 2000; Yanagi et al., 2006). It is generally accepted that, whereas laboratory and vaccine strains use both CD150 and CD46 as their cellular receptors, wild-type MV strains mainly use CD150.

Oncolytic viruses such as MV have been selected or engineered to replicate in tumour cells. Approaches towards specifically targeting cancer cells exploit mutations in multiple genes contributing to the malignant phenotype as insufficient antiviral defence programmes, constitutive activation of signalling pathways, transcriptional programmes, or expression of antigens that are unique to or are overexpressed on the surface of tumour cells. As cell-surface recognition and virus entry is the key first step for specific targeting, engineering oncolytic viruses in order to recognize exclusively the tumour cell-surface is mandatory. Therefore, retargeting of oncolytic viruses including MV is an obvious and promising approach to exploit the potential of MV in virotherapy (Parato et al., 2005). As a proof of principle, recombinant MV that has been blinded for usage of CD46 and/or CD150 by site-specific mutagenesis within the H protein sequence has been rescued successfully, as have recombinants retargeted for usage of carcinoembryonic antigen or CD38 by fusion of the H protein open reading frame with that of the respective single-chain antibody (scFv) (Nakamura & Russell, 2004; Parato et al., 2005). For multiple myeloma (MM) cells in particular, several candidate surface antigens including MUC-1, sperm protein 17 and differentiation antigens such as CD38 and CD138 have been evaluated for targeted therapy, although with limited success to date.
Recently, a mouse monoclonal antibody (mAb), termed Wue-1, was generated after immunization using the pulmonary low-grade mucosa-associated lymphoid tissue-type B-cell lymphoma cell line H3302 (Greiner et al., 2000a). In the current study, we have shown that this antibody exclusively stains well-differentiated B cells and their malignant counterparts, such as healthy plasma cells, primary MM cells and immunocytoma cells. We genetically engineered and rescued a CD46- and CD150-blinded recombinant MV and retargeted it for Wue-1+ cells by fusion of the sequence encoding an scFv derived from Wue-1 (scFv-Wue) C-terminal to the blind H protein reading frame (MV-Wue). The retargeted MV-Wue could be propagated in primary MM cells to similar virus titres as non-retargeted virus. In agreement with its receptor specificity, MV-Wue specifically and efficiently infected primary MM cells and induced apoptosis.

**RESULTS**

**mAb Wue-1 specifically binds MM cells that express CD46, but not CD150, as natural MV receptors**

As a prerequisite for selective targeting, the restricted binding pattern of Wue-1 mAb was re-evaluated (Greiner et al., 2000a). As revealed in this considerably larger series of immunostainings and flow cytometry analysis with a variety of samples of haematological malignancies and healthy haematological and non-haematological tissues, the mAb Wue-1 bound to almost 100% of primary MM
samples (50/51 samples) and Waldenstrom’s disease samples (14/15) (shown in Fig. 1a and d) and, as expected, to healthy plasma cells. In contrast, no binding could be detected to a wide range of non-plasma-cell-associated healthy tissues or to other B-cell malignancies apart from Waldenstrom’s disease. The various B-cell malignancies were specially chosen to demonstrate the highly restricted binding pattern throughout the corresponding stages of B-cell differentiation from immature B-cell malignancies to MM cells (Table 1). In addition, Wue-1 reactivity was not influenced by the therapeutic history of the patient. In contrast, CD46, the high-affinity receptor for attenuated MV strains, was readily detectable to almost identical levels on primary samples of CD138⁺ malignant plasma cells (Fig. 1b), the heterogeneous group of the CD138⁻ fraction (data not shown) and B cells used as controls (Fig. 1b). As expected, these also expressed the second MV receptor, CD150, whereas primary MM samples tested by flow cytometry showed no surface expression of CD150 (Fig. 1c).

In conclusion, labelling experiments of different haematological malignancies and healthy haematological and non-haematological tissues with Wue-1 confirmed a restricted binding pattern of this antibody, which selectively bound malignant and normal plasma cells. From these data, it could also be concluded that Wue-1 only binds to very mature healthy or malignant B cells and plasma cells.

**Table 1. Labelling with mAb Wue-1 is restricted to haematological malignancies with plasma cell differentiation**

<table>
<thead>
<tr>
<th>Type of lymphoma</th>
<th>No. positive/total</th>
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<tbody>
<tr>
<td>Plasmacytoma/myeloma</td>
<td>50/51</td>
</tr>
<tr>
<td>Waldenstrom’s disease</td>
<td>14/15</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>1/13</td>
</tr>
<tr>
<td>Follicular centre lymphoma</td>
<td>0/23</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>0/10</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>0/5</td>
</tr>
<tr>
<td>B-cell lymphocytic leukaemia</td>
<td>0/13</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>0/21</td>
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MV-Wue was generated as described in Methods. Fig. 2(a) depicts the recombinant MV-Wue.

In addition to the restricted binding pattern of Wue-1, efficient replication of MV in target cells represents another important aspect of the therapeutic profile of MV-Wue (Parato et al., 2005). To investigate whether the replication efficiency was affected by the genetic modifications, one-step growth curves were performed using Vero-His cells expressing both the CD46 antigen and an anti-His-tag scFv. We compared MV-EGFP, which had the parental H tropism for CD46 and CD150, with MV-Wue, which had the mutated H protein modified to display scFv-Wue and the His tag. Displaying scFv-Wue clearly did not interfere with replication of the virus, as similar final titres were obtained for MV-EGFP and MV-Wue for both cell-associated and cell-free virus (10⁶ TCID₅₀ ml⁻¹) (Fig. 2b).

Efficient replication in a highly susceptible cell line additionally expressing an anti-His-tag scFv may allow efficient replication of MV *in vitro*, but may not be sufficient for its clinical application where the virus would have to replicate in primary MM cells. We thus analysed whether MV-Wue could infect primary MM cells known to support MV replication (Dingli et al., 2004; Nakamura & Russell, 2004). In these infection experiments, Wue-1⁺/CD138⁺ cells were used (CD138⁺ primary myeloma cells). As expected, these cells could be infected effectively with the retargeted virus MV-Wue as indicated by EGFP expression (Fig. 3a, left panel). In contrast, the CD138⁻ fraction of the same bone marrow sample and primary B cells did not support entry of the retargeted virus MV-Wue (Fig. 3a, middle and right panels). The requirement for Wue-1 for entry of the retargeted virus into malignant cells was further supported by inhibition of MM cell infection by pre-treatment with Wue-1 mAb resulting in an approximate 60 ± 10 % reduction in EGFP expression (Fig. 3b).
Pre-incubation with an IgG control antibody did not influence the percentage of infected malignant plasma cells by MV-Wue. As a positive control, MV-EGFP pre-incubation resulted in 100% infected MM cells independently of pre-incubation of the target cells with Wue-1 or IgG control antibody (data not shown). It is of note that cells that expressed EGFP always co-expressed CD138 when MV-Wue was used (see right panels in Fig. 3b).

In the next step, we investigated whether MV-Wue infection of primary MM cells resulted in full replication of the retargeted virus. Fig. 4(a) shows the results of a comparison of titres of mock-infected (not shown), MV-EGFP-infected and MV-Wue-infected primary MM cells determined at different time points in the absence or presence of the Wue-1 antibody or an IgG control antibody. As shown in Fig. 4(a), upper panels, MV-EGFP produced increasing virus titres replicating in primary MM cells independently of the presence of Wue-1 mAb or the IgG control antibody. As shown in Fig. 4(a), upper panels, MV-EGFP produced increasing virus titres replicating in primary MM cells independently of the presence of Wue-1 mAb or the IgG control antibody. In contrast, MV-Wue was able to replicate equally efficiently in MM cells in the absence of antibodies or in the presence of the IgG control antibody, but the virus titre was considerably reduced when the primary MM cells were pre-incubated with mAb Wue-1 (Fig. 4a, lower panels). In summary, MV-Wue was able to replicate as efficiently as its non-retargeted counterpart in primary MM cells, and cell entry was mediated by specific interaction of the virus with its target receptor.

As MV has a high tropism for monocytes and their precursors, we next focused on characterizing the effects of MV-EGFP and MV-Wue on CD33+ cells, a marker that defines myeloid cells and myeloid progenitor cells. As shown in Fig. 4(b), we compared primary MM cells (CD138+) and myeloid progenitor cells (CD33+) from the same patient after magnetic bead selection and incubation at day 3.5 after infection with MV-EGFP or MV-Wue, or mock infection. Irrespective of prior antibody pre-incubation, MV-EGFP incubation led to a strong reduction in both CD138+ malignant plasma cells and CD33+ cells (Fig. 4b). In contrast, MV-Wue reduced the number of CD138+ cells, but did not diminish the number of CD33+ cells significantly, when compared with mock-infected CD33+ cells (Fig. 4b). Incubation of the target cells with IgG control antibody did not result in a significant difference in cell loss of either CD138+ or CD33+ cells after MV-Wue infection. However, pre-treatment of the CD138+ cells with Wue-1 did strongly reduce elimination of the malignant plasma cells by MV-Wue (Fig. 4b). Further measurements for EGFP positivity by FACS revealed that CD138+ and CD33+ cells were positive for EGFP expression when incubated with MV-EGFP (Fig. 4c, upper panels). This was also the case for CD138+ cells, but not for CD33+ cells when treated with MV-Wue (Fig. 4c, lower panels).

In addition to the observed specificity of MV-Wue for CD138+, but not CD33+, cells, lymphocytes were not affected by MV-Wue but were greatly diminished by MV-EGFP infection in bone marrow samples of MM patients (data not shown). Thus, MV-Wue was highly specific for CD138+ cells and did not cause a significant reduction in other haematopoietic cells.

Fig. 2. (a) Full-length genome of the retargeted MV-Wue displaying the scFv-Wue. A single-chain antibody based on the mAb Wue-1, scFv-Wue, was attached to the C terminus of a mutated H protein that was incapable of interacting with CD46 or CD150. EGFP was encoded in the full-length genome of MV-Wue and served as a marker for virus replication in target cells. Additionally, MV-Wue expressed a 6His tag necessary for propagation of the retargeted mutated virus in Vero-His cells displaying an anti-6His-tag scFv. (b) Virus titres of MV-EGFP and retargeted MV-Wue were similar in Vero-His cells. Vero-His cells were infected with MV-EGFP (filled bars) and MV-Wue (shaded bars) at an m.o.i. of 2. Supernatants and cells were harvested at 24, 36, 48 and 72 h p.i. and titrated on Vero-His cells. Experiments were replicated ten times and results are shown as means ± SD.
Specific infection of primary MM cells by MV-Wue results in induction of apoptosis

Primary MM cells supported the entry and replication of MV-Wue. To investigate the fate of these infected malignant plasma cells, induction of apoptosis was investigated by Annexin V staining. In Fig. 5, a representative primary bone marrow sample of a patient containing almost 100% plasma cells is depicted. At 72 h after infection of the primary plasma cells, both MV-EGFP (Fig. 5a, b) and MV-Wue (Fig. 5c) induced a high degree of Annexin V positivity in PI-negative cells expressing EGFP (EGFP positivity not shown) compared with mock-infected cells (purple area). Pre-incubation of these cultures with Wue-1 resulted in a significant reduction in Annexin V-positive cells (data not shown). This was not the case when MV-EGFP was used (Fig. 5b). Incubation of the target cells with IgG control antibody did not influence the percentage of cells positive for Annexin V or the cell counts (Fig. 5a, c). The results of Annexin V staining without antibody
pre-incubation of the MM cells were similar to those when pre-incubated with IgG control antibody (data not shown). Thus, entry and replication of MV-EGFP and of retargeted MV-Wue into malignant plasma cells correlated with positive Annexin V labelling of PI-negative cells. Together with the observed rapid cell loss, these data provide evidence that MV-Wue can selectively induce programmed cell death in malignant plasma cell samples.

**DISCUSSION**

MV Edmonston (MV-Edm)-derived strains possess an array of advantageous characteristics making them suitable candidates to be tailored as a tool to specifically kill target malignant cells. The virus life cycles of native and modified MV allow efficient replication, reaching comparable titres, levels of cytolysis and cell-to-cell spread, protecting MV from immune responses. The availability of a recombinant system allows genetic modification of MV to retarget it to tumour antigens selectively expressed on malignant cells. The stability and non-recombination of the modified virus, even after many replication cycles, allow constant alterations and a favourable safety profile of MV-Edm-based strains and especially of modified virus variants unable to use CD46 and CD150 as receptors, preventing unwanted damage in tissues positive for these two known natural MV receptors (Grote et
al., 2001; Nakamura & Russell, 2004; Parato et al., 2005; Zuniga et al., 2007). In contrast to non-replicating or non-viral vector systems, MV-Edm, as a replicating virus, offers an increased oncolysis. MV-Edm exhibits a considerable local bystander effect, capable of destroying tumour cells by cell-to-cell fusion with cells that the virus is unable to reach initially. Therefore, MV-Edm can achieve significant neoplastic tissue destruction without necessarily transducing all tumour cells. It is of note that MV-Edm retains this considerable oncolytic activity after genetic modification, especially after modifications to retarget the virus.

As oncolytic virotherapy has been greatly limited by lack of knowledge of the determinants of viral tropism and of ways of working with these determinants to generate viruses that are more specific for cancer cells, a great deal of effort has been made towards understanding the interplay between target cells and viruses at the molecular level. It has been

Fig. 4. (a) MV-Wue and MV-EGFP produce similar titres on primary MM cells. Primary MM cells were mock-infected or infected with MV-EGFP (upper panels) or MV-Wue (lower panels). Supernatants and cells were harvested at 24, 48 and 72 h p.i. and titrated on Vero-His cells. Virus titres of MV-EGFP and MV-Wue increased over time (open bars), demonstrating that both viruses can complete a whole replication cycle in primary MM cells. To address the question of whether infection and replication of MV-Wue is mediated through the specific interaction of the incorporated scFv-Wue and its ligand on MM cells, the same propagation experiment was performed after treatment of the MM cells with Wue-1 antibody (filled bars) or with an IgG control antibody (shaded bars). Under these conditions, MV-EGFP titres were not affected by either antibody treatment, whereas MV-Wue titres decreased significantly when MM cells were treated with Wue-1 mAb prior to infection. Experiments were replicated ten times and results are shown as means ± S.D. (b) MV-Wue specifically eliminates CD138+ cells, but not CD33+ myeloid progenitor cells. CD138+ and CD33+ cells from the same MM patient in all experiments were mock-infected (open bars) or infected with MV-EGFP (shaded bars) or MV-Wue (filled bars), and cell counts were determined at 3.5 days p.i. by FACS analysis. The numbers of CD138+ cells were strongly decreased for both MV-Wue and MV-EGFP (left panel, open bars). CD33+ cells were reduced only for MV-EGFP infection, and not for MV-Wue infection when compared with mock-infected cells (right panel, open bars). Pre-incubation of CD138+ or CD33+ cells with an IgG control antibody did not influence these results, but pre-incubation with Wue-1 significantly reduced CD138+ cell loss caused by MV-Wue. CD33+ cell numbers were not decreased by MV-Wue (right panel). Experiments were replicated five times and results are shown as means ± S.D. (c) CD138+ and CD33+ cells incubated with MV-EGFP expressed EGFP, whereas, after incubation with MV-Wue, only CD138+ cells were positive for EGFP. FACS analysis for EGFP expression in CD138+ and CD33+ cells after incubation with MV-EGFP or MV-Wue showed that MV-EGFP infected both cell populations, but MV-Wue only infected CD138+ cells, resulting in EGFP−CD33+ cells and EGFP+CD138+ cells. Experiments were replicated five times.

Fig. 5. MV-Wue-infected primary myeloma cells stain positive for Annexin V. A sample of primary plasma cell leukaemia cells (>99 % positive for CD138) was mock-infected or incubated with MV-EGFP (a, b) or MV-Wue (c, d) at an m.o.i. of 0.5 after pre-incubation with mAb Wue-1 or an IgG control antibody as indicated. All histograms showed PI-negative cells after incubation with the antibodies and viruses. As shown in (a) and (b), the vast majority of the PI-negative cells infected with MV-EGFP showed binding of Annexin V–Cy5 (green line) compared with the corresponding population that were mock-treated (purple area), irrespective of pre-incubation with the Wue-1 or IgG control antibody. Using MV-Wue for infection, Wue-1 pre-incubation strongly reduced the fraction of Annexin V-positive cells (reduction of 80±16 %) (d), whereas IgG control antibody pre-incubation did not diminish the percentage of Annexin V-positive cells (c). Experiments were replicated five times.
shown that cancer cells possess a high potential for successful replication of naturally oncolytic viruses, whereas non-transformed cells can control virus infection and replication better. As more extensive testing has identified limited efficacy or dose-limiting toxicities, the need to improve the characteristics of naturally oncolytic viruses has become clear. Therefore, research has moved towards retargeting strategies to increase the specificity for malignant cells (Cattaneo et al., 2008).

In this report, against the background of data that mAb Wue-1 binds specifically to healthy plasma cells and MM cells irrespective of treatment status, a new retargeted MV was constructed, MV-Wue, using an H protein variant that had been mutated with regard to its interaction with CD46 and CD150 and a scFv based on mAb Wue-1, which was attached to this mutated H protein. MV-Wue could be propagated efficiently in primary MM cells, which are highly susceptible to MV-Edm derivates, and it was able specifically to infect and kill the primary malignant plasma cells by apoptosis. As mentioned above, a remarkable observation during testing of the Wue-1 antibody and the retargeted MV-Wue was that neither the number of previous chemotherapies nor the treatment modality (stem-cell transplantation or conventional chemotherapy) negatively influenced the interaction of Wue-1 mAb or MV-Wue with the primary malignant plasma cells.

As shown previously by Leonard et al. (2008), retargeted viruses in particular can be reasonably characterized based on their function rather than on the interacting molecule. Additionally, coherent functional data in vitro should be the basis for further evaluation of MV-Wue in animal models, including undesired side effects and therapeutic potential in the near future, even without exact knowledge of the antigen recognized by Wue-1 mAb or scFv-Wue incorporated in MV-Wue. However, given the consistent functional data together with the immunohistochemical data in this study, which showed that the expression pattern of the antigen recognized by Wue-1 is distinct from known CD antigens and published antibodies in the differentiation pathway of normal B and plasma cells (Greiner et al., 2000a), it is interesting to define the corresponding antigen recognized by MV-Wue. So far, the most obvious approaches, including expression libraries, have failed to clarify the identity of the Wue-1 antigen, but further efforts are under way to characterize this antigen in order to enrich our knowledge of the relevant interactions between this potential oncolytic virus and the proteins of the target cell.

The data generated in this report concerning unwanted infection of healthy cells showed no discrepancy between the immunohistochemical data and cells infected by MV-Wue tested so far. Primary CD33+ cells, which have been described as highly susceptible to MV infection, and primary B cells as plasma-cell precursors were used in infection experiments as negative controls and were not infected with MV-Wue. As the frequency of primary plasma cells in the peripheral blood of healthy donors is very low, we did not test this population in our experiments with the retargeted virus. However, against a background of widespread use of the anti-CD20 mAb rituximab in lymphoma therapy, which is well tolerated although its administration is followed by a long-lasting B-cell depletion, the partial B-cell defect by selectively eliminating plasma cells after the application of MV-Wue should generate no relevant toxicity problems.

In order to develop a potent oncolytic tool, successful functional tests in vitro using susceptible cell lines or primary cells should lead directly to evaluation of the efficacy of tumour reduction in vivo in animal models wherever possible using reporter or tracker genes with objective measurable parameters of safety and efficacy. To maximize the oncolytic potential, the retargeted virus can be combined with pro-drug convertases to generate synergistic effects with chemotherapeutic drugs in clinical use and should be protected from the patients’ immune system (Iankov et al., 2007; Ong et al., 2007; Cattaneo et al., 2008).

In conclusion, MV tolerated well the attachment of scFv-Wue at the C terminus of its H protein as an independently folding protein. This did not significantly impair its replicative capacity in primary MM cells, but rather enabled the engineered virus to infect and kill defined cells via a new interaction using scFv-Wue specific for MM cells.

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