Measles virotherapy in a mouse model of adult T-cell leukaemia/lymphoma

Cecilia Parrula,1 Soledad A. Fernandez,2,3 Bevin Zimmerman,1 Michael Lairmore1,3,4 and Stefan Niewiesk1,3,4

1Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio, USA
2Center for Biostatistics, The Ohio State University, Columbus, Ohio, USA
3Comprehensive Cancer Center, The Arthur James Cancer Hospital and Research Institute, The Ohio State University, Columbus, Ohio, USA
4Center for Retrovirus Research, The Ohio State University, Columbus, Ohio, USA

Adult T-cell leukaemia/lymphoma (ATL) is a highly aggressive CD4+ T-cell malignancy caused by human T-cell leukaemia virus type 1. Measles virus (MV) oncolytic therapy has been reported to be efficient in reducing tumour burden in subcutaneous xenograft models of lymphoproliferative disorders such as myeloma, B-cell lymphoma and cutaneous T-cell lymphoma, but its potential to reduce tumour burden in disseminated lymphoproliferative disorders such as ATL remains to be determined. In this study, MV oncolytic therapy was evaluated in the MET-1/NOD/SCID xenograft mouse model of ATL. Treatment with the vaccine-related strain MV-NSE led to a significant reduction in tumour burden. In mice with a high tumour burden, therapy with MV-NSE significantly increased survival beyond any other single treatment tested previously using this model. Interestingly, signs of morbidity (cachexia) in mice treated with MV were not directly associated with tumour burden, but were correlated with the secretion of interleukin-6 by MET-1 cells and host cells. The results suggest that MV therapy could be a promising therapy for generalized lymphoproliferative disease.

INTRODUCTION

Chronic infection of CD4+ T-cells in subjects infected with human T-cell lymphotropic virus type 1 (HTLV-1) results in adult T-cell leukaemia (ATL) in 3–5% of infected individuals. In the acute form of the disease patients exhibit generalized lymphadenopathy, hepatosplenomegaly and infiltration of skin and various organs. ATL patients often have calcium mobilization from bone and hypercalcaemia, which has been associated with secretion of parathyroid hormone related protein (PTHrP), tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) and IL-6. Patients with acute forms of ATL have a poor prognosis owing to the lack of an effective therapy against the cancer, and as a result survival times of patients range from 9 to 24 months (Zhang et al., 2005). To test novel therapeutic approaches against ATL, a xenograft mouse model has been established by intraperitoneal inoculation of patient-derived ATL cells (MET-1) into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Zhang et al., 2002a). In this model, tumour burden correlated with secretion of the α chain of the IL-2 receptor (sIL-2Rα). Based on sIL-2Rα levels, tumour burden has been classified as low (1–10 ng ml−1) or high (20–70 ng ml−1) (Zhang et al., 2002a). An advantage of this animal model is the multicentric spread of MET-1 cells with perivascular invasion of several organs including spleen, liver, pancreas, gastrointestinal tract, uterus, heart and lung (Parrula et al., 2009). In the serum of mice with MET-1 tumours, parathyroid hormone related protein (PTHrP) and IL-6 are detectable (but not IL-1 or TNF-α) (Parrula et al., 2009) and they develop hypercalcaemia similar to ATL patients. The MET-1/NOD/SCID mouse model has been used successfully to evaluate antibody-based treatments for ATL preclinically (Chen et al., 2009; Zhang et al., 2002a, 2003a, b, 2005).

An agent used experimentally to treat T-cell proliferative diseases is measles virus (MV). In nude mice, the subcutaneous inoculation of cutaneous T-cell leukaemia (CTCL) cells led to the formation of solid tumours, which, upon intratumoral injection with MV, regressed within 2 weeks (Künzi et al., 2006). In patients with CTCL, MV given by intratumoral injection showed a similarly beneficial effect (Heinzerling et al., 2005). Whether MV application in T-cell malignancies with multicentric engraftment like ATL will be as successful as in the treatment of localized malignancies still needs to be determined. In vivo, the oncolytic effect of MV therapy is thought to be because of the combination of cell
destruction through fusion and apoptosis, and increased tumour immunogenicity owing to upregulation of heat-shock proteins (HSP) after MV infection (Grote et al., 2001). MV infection induces expression of HSP72, which in turn increases MV transcription and replication by interacting with the unstructured carboxy terminus of the MV nucleocapsid protein (Zhang et al., 2005). Expression of HSP72 subsequent to MV infection stimulates the immune system by stimulating natural killer (NK)-cell activity (Taglia et al., 2008; Segal et al., 2006) and enhancing tumour-antigen presentation to professional antigen presenting cells (Segal et al., 2006).

Here, we tested the effect of measles oncolytic therapy in the MET-1 NOD/SCID mouse model of ATL. Therapy with a vaccine-related strain of MV (MV-NSE) was capable of markedly reducing tumour burden (independent of the initial degree of tumour burden), and increasing survival by 40–47 days. Interestingly, MV was capable of controlling leukaemic cells at very low levels even after cessation of treatment. Treatment with MV-HSP72 increased survival time relative to therapy with MV by 3 days. Ultimately, mice did not die of resurgent leukaemia but cachexia due to IL-6 secretion. Collectively, these data suggest MV therapy as a promising viral oncolytic therapy in generalized T-cell lymphoproliferative disease, such as HTLV-1 ATL, and identify potential cytokine complications to address for further preclinical development of this approach.

RESULTS

MV treatment efficiently reduces low and high tumour burden in leukaemic mice

Studies evaluating the potential of MV therapy in T-cell proliferative diseases have so far been limited to localized cutaneous T-cell lymphoma, with encouraging results (Künzi et al., 2006; Heinzerling et al., 2005). Herein, we tested the effect of MV therapy in a multicentric xenograft model of ATL. Three weeks after inoculation of 2 × 10⁶ MET-1 cells, the NOD/SCID mice engrafted had a low level of tumour burden as measured by sIL-2Rα (5 ± 4 ng ml⁻¹) in serum. Subsequently, mice were injected intraperitoneally once a week for 5 weeks with MV-NSE (10⁵ p.f.u. per mouse). In control animals, sIL-2Rα concentration increased over time to reach high levels (120 ± 70 ng ml⁻¹) at the time of euthanasia. In MV-treated animals, there was a reduction of >95% in sIL-2Rα concentration (Fig. 1) to undetectable levels after only two treatments, which remained undetectable-to-low (170 ± 350 pg ml⁻¹) during the rest of the treatment (weeks 5–8) until the time of euthanasia in week 9. After euthanasia in week 9, the organs of control and treated animals were evaluated by histology. Histopathological analysis of tissues from control animals showed minimal infiltration (for grading see Methods) of leukaemic cells in pancreas, large intestine, spleen, uterus and heart. Mild infiltration was seen in brain and kidney, and moderate infiltration in lung and adrenal gland. In animals treated with MV-NSE, leukaemic cell infiltration was limited to minimal infiltration of liver and lung, and mild infiltration of the heart. In a single mouse treated with MV-NSE, large multinucleated cells (syncytia) were found in a mediastinal lymph node. Immunohistochemical staining, with an antibody specific for human Ki67 that does not cross-react with mouse Ki67, confirmed the cells to be human cells, and antibody staining for MV demonstrated the syncytia to be infected with MV (not shown).

To analyse the potential of MV oncolytic therapy further, MV treatment was assessed in mice with a high tumour burden. Mice that had developed a high tumour burden (sIL-2Rα, 180 ± 65 ng ml⁻¹) 6 weeks after inoculation of the MET-1 cells were treated once a week for 4 weeks with MV-NSE (10⁵ p.f.u. week⁻¹ per mouse). In control animals, serum sIL-2Rα concentration progressively increased (Fig. 2) until the animals were euthanized according to humane end point criteria (see Methods) between weeks 8 and 10 of the trial. In MV-NSE-treated animals, two treatments were sufficient to markedly reduce tumour burden by approximately 88%, to a serum concentration of sIL-2Rα of 20 ± 19 ng ml⁻¹. Serum sIL-2Rα concentrations remained low (16 ± 9 ng ml⁻¹) during the time of the remaining two treatments. After the end of the last treatment with MV-NSE

![Fig. 1. sIL-2Rα concentrations in sera of mice with small tumour burden with and without MV treatment. Three weeks after inoculation with tumour cells, mice (n=5) were treated weekly with MV-NSE intraperitoneally for 5 weeks (arrows indicate treatments) at a dose of 10⁵ p.f.u. week⁻¹ per mouse or left untreated (control). Both groups were euthanized 1 week after the last treatment (week 9). Concentration of sIL-2Rα progressively increased in control animals (○), while in treated animals (●) concentration decreased and remained low until euthanasia. The concentrations of sIL-2Rα in both groups are statistically significantly different and the trends of the two groups over time are different (P<0.001, mixed linear regression).](http://vir.sgmjournals.org)
(week 10), sIL-2Rx concentration started to rise, until it reached a peak on week 12 (230±72 ng ml⁻¹), and then dropped to a significantly lower level (93±46 ng ml⁻¹). Treatment with MV-NSE increased survival by at least 3 weeks. MV-NSE-treated animals were euthanized between weeks 13 and 17, while all mice in the control group were euthanized by week 10. Interestingly, mice had to be euthanized not because of high tumour burden, as judged by low levels of sIL-2Rx, but because of constant weight loss, which started after the cessation of treatment and continued until they had to be euthanized.

**MV treatment is effective after cessation of treatment**

To clarify the reason for the drop in tumour burden as determined by sIL-2Rx after week 12 (Fig. 2), mice were evaluated between weeks 12 and 13. Histopathology showed mild-to-moderate infiltration of the lung and moderate-to-marked infiltration of the heart by leukaemic cells in control groups as well as in MV-treated animals. In the leukaemic infiltrates of MV-treated animals, however, numerous syncytia were seen in the lung and heart of six of the seven animals (Fig. 3). Immunohistochemistry with antibodies specific for human Ki67 and MV nucleocapsid showed that the syncytia had a human origin and were infected with MV, respectively (Fig. 3). Leukaemic cells and syncytia were also found in the subcutis of two animals and in the mediastinum of one animal. The absence of MV in the spleen was confirmed by quantitative PCR (data not shown).

**Long-term survivors have increased concentrations of human and mouse IL-6 in serum**

As mentioned before, mice in the MV-treated group did not succumb because of high tumour burden but had to be euthanized because of chronic weight loss. In mice, weight loss has been observed after secretion of TNF-α and IL-6 (Argilés & López-Soriano, 1999), either by the human leukaemic cells or in response to the heterologous cells by the mouse immune cells (Barton & Murphy, 2001). In cancer patients, the pro-inflammatory cytokines TNF-α and IL-6 have also been implicated in the induction of cachexia. We have shown previously that MET-1 cells, after inoculation into mice, secrete human IL-6 (hIL-6) but not human TNF-α (Parrula et al., 2009). For these reasons, the levels of human and mouse IL-6 and mouse TNF-α were measured by ELISA in serum from control (untreated) and MV-treated mice. Pooled sera from the control group contained 37 pg ml⁻¹ hIL-6 at the beginning of treatment, which increased to 320 pg ml⁻¹ at study termination. This increase paralleled the increase observed for sIL-2Rx concentration. In the MV treatment group, the concentration of hIL-6 (6–42 pg ml⁻¹) also correlated with the concentration of sIL-2Rx (Fig. 4a). An increased concentration of mouse IL-6 (mIL-6) was found in the serum of mice with MET-1 tumours, but not in the serum of naive NOD/SCID mice. Similar concentrations of mIL-6 were found in the serum of control (9.8±8.0 pg ml⁻¹) and MV-treated (8.6±6.2 pg ml⁻¹) mice with MET-1 tumours (Fig. 4). Concentrations of mIL-6 were therefore dependent on the presence of leukaemic cells but were independent of the magnitude of tumour burden. No mouse TNF-α was found in any of the samples by ELISA (data not shown). An alternative explanation for cachexia might be functional impairment of organs infiltrated by tumour cells. However, in MV-treated animals tumour cell infiltration was confined to lung and heart, with minimal-to-mild infiltration in the lung and mild-to-moderate infiltration in the heart (not shown). The level of infiltration was not sufficient to cause chronic heart disease (supported by the absence of signs of congestion in the liver and lung) and related cachexia. In contrast, untreated control animals had moderate-to-marked infiltration of pancreas, liver, spleen, kidney, ovary, large and small intestine, adrenal, heart, lung and brain (not shown).

**Production and characterization of MV expressing HSP72 in vitro**

The results from the experimental treatment with MV indicated that the oncolytic potential of MV leads to strong reduction but not complete eradication of leukaemic cells. In order to improve treatment, a recombinant virus which expresses HSP72 was produced. Increased expression of
cellular HSP72 has been reported to upregulate MV gene expression and MV replication (Vasconcelos et al., 1998). These findings suggest that a recombinant MV expressing HSP72 (MV-HSP72) would replicate faster and consequently have a greater oncolytic effect. In addition, cells infected with MV-HSP72 should express HSP72 on the cell surface and thus should promote NK-cell binding and resulting cytolysis (Taglia et al., 2008). To test this hypothesis, MV-HSP72 was produced by insertion of the HSP72 gene with a vesicular stomatitis virus (VSV) tag between the haemagglutinin and polymerase genes of a molecular clone of MV-NSE (Fig. 5a), similar to previous published molecularly cloned viruses (Singh & Billeter, 1999). HSP72 expression was verified from lysates of infected Vero cells by Western blotting (Fig. 5b). Basal HSP72 expression in uninfected cells was similar to expression in cells infected with MV-NSE. In comparison, cells infected with MV-HSP72 expressed the recombinant HSP72 (as demonstrated by staining for the VSV tag) and both endogenous and virally expressed HSP72 led to an approximately twofold increase in HSP72 levels (Fig. 5b).

To compare MV and MV-HSP72 replication, titres of cell-associated virus and virus released into supernatant (cell-free virus) were compared over a time course (Fig. 5c). The growth curves of the viruses did not differ from each other. To test the lytic potential of MV-HSP72 versus MV-NSE in vitro, MT-2 cells (human CD4+ T-cells transformed by HTLV-1) were infected with MV and MV-HSP72, and counted 24, 48 and 72 h later. Infection with either virus led to a significant reduction in cell number over 3 days (Fig. 6a). What was the statistical test used to calculate this significant result?

However, no difference in cell number was found between infections with MV-HSP72 and infections with MV-NSE. HSP72 expression on the surface of cells stimulates cytolysis by NK cells (Taglia et al., 2008). To test whether HSP72 expression was increased after infection with MV-HSP72, Jurkat cells (a human CD4+ leukaemic T-cell line) were infected with MV-NSE and MV-HSP72, and surface HSP72 expression was determined by flow cytometry 48 h post-infection (Fig. 6b). Surface expression of HSP72 was significantly greater after infection with MV-HSP72 than with MV-NSE (P=0.017).

No clinically relevant increase in treatment efficacy after in vivo treatment with MV-HSP72

In addition to a direct cytolytic effect, MV-HSP72 should also be able to activate NK cells because of increased expression of HSP72 on infected leukaemic cells. NOD/SCID mice lack...
B- and T-cells, but have detectable levels of NK-cell cytotoxic activity (Shultz et al., 2003). In vivo treatments with MV-NSE and MV-HSP72 were compared in animals with a high tumour burden (sIL-2Ra, 170 ± 67 ng ml⁻¹). Animals were treated four times at weekly intervals with either MV-NSE or MV-HSP72 (10⁶ p.f.u. per treatment) or left untreated (control), and subsequently tumour development was followed until the mice had to be euthanized in accordance with humane end-point criteria (weight loss of ≥ 20% of body weight). The estimated mean survival times were: 24 days in the control group; 64.4 days in the MV-treated group; and it was prolonged to more than 67.6 days in the MV-HSP72-treated group. One of the five mice in the MV-HSP72 treatment group was still alive at day 81. Although the statistical comparison of time of survival between the MV-NSE- and MV-HSP72-treated groups (Fig. 7) determined that survival was significantly higher in MV-HSP72 treated animals (P = 0.0183), the clinical relevance of this finding is questionable. As for the previous large tumour burden experiment, morbidity in MV-NSE- and MV-HSP72-treated groups was associated with the development of cachexia and at the time of death animals from both groups had the same (low) tumour burden as determined by serum sIL-2Rα concentrations and histopathological analysis (data not shown).

**DISCUSSION**

Our data demonstrate that MV oncolytic therapy was effective in the MET-1/NOD/SCID xenograft mouse model of ATL. Treatment with MV and MV-HSP72 was efficient in reducing tumour burden by approximately 90% even at high tumour burden. Approximately 2 weeks after the last treatment with MV, a resurgence of leukaemic cells was countered by increased MV replication. This was evidenced by the presence of numerous MV-infected syncytia, which caused a drop in tumour burden, as determined by levels of sIL-2Rα and histopathological analysis. This is a very interesting finding that has not been reported in previous preclinical trials evaluating MV therapy.

Independent of the level of tumour burden, our data presented herein indicate that after just two treatments with MV at a dose of 10⁶ p.f.u., tumour burden decreased markedly. This contrasts with the results of MV therapy in B-cell malignancies [B-cell lymphoma (Grote et al., 2001) and myeloma preclinical studies (Peng et al., 2001)] where doses of 10⁷ p.f.u. were necessary for tumour regression. Whether these differences are cell-type specific or depend on the characteristics of the specific cell line, remains to be evaluated.

The inoculation of ATL cells (MET-1) into NOD/SCID mice has been used to model multicentric engraftment of leukaemic cells and to develop therapeutic approaches based on treatment with antibodies against molecules expressed on the surface of ATL cells such as CD2, CD25 and CD52 (Chen et al., 2009; Zhang et al., 2002a, 2003a, b, 2005). Overall, antibody treatment was effective when used with low tumour burdens. However, efficacy decreased with increasing tumour burden. In contrast, treatment with MV and MV-HSP72 was efficient in reducing even high tumour burden by approximately 90% and prolonged survival beyond any antibody treatment (Zhang et al., 2002a, 2005). As antibodies are particularly effective in the presence of low tumour burden, it would be of interest in future studies to combine MV virotherapy with antibody treatment to eliminate residual tumour burden.
Although treatment with MV decreased tumour burden to very low levels and significantly increased survival, mice eventually developed cachexia and had to be euthanized. This cachexia was correlated with the secretion of hIL-6 by leukaemic cells and IL-6 by mouse cells. Serum hIL-6 concentrations paralleled changes in tumour burden detected by sIL-2Rα because both molecules are secreted by MET-1 cells, even though hIL-6 concentrations were 1000–10,000-fold lower than sIL-2Rα concentrations. In mice treated with MV where tumour burden was very low, hIL-6 concentrations were in the range 6–32 pg ml⁻¹. These concentrations are in the same range of concentrations reported for human cancer patients who developed cachexia attributed to IL-6 secretion (Kuroda et al., 2007). Therefore, because hIL-6 is active on murine cells (Evans et al., 1993) and supported by data from cancer patients (Kuroda et al., 2007) and other human xenograft mouse models (Barton & Murphy, 2001), the constant secretion of hIL-6 by MET-1 cells and secretion of mIL-6 by mouse immune cells is the most likely cause of the development of cachexia.

In a treatment experiment, MV-HSP72 did not increase survival by a clinically relevant margin. The rationale for the use of MV-HSP72 was that this virus would have a greater oncolytic effect (reflected by increased survival), because of the effect of HSP72 expression on viral replication and on cells of the immune system. The expression of HSP72 enhances MV gene expression and consequently MV replication in tissue culture and in transgenic mice. However, the analysis of growth curves showed no differences in replication between MV and MV-HSP72. Recently, it has been reported that HSP40 (Couturier et al., 2009) is involved as a co-chaperone in the replication of MV with a stoichiometric ratio of 10:1 (HSP72 : HSP40). It is possible that a recombinant MV expressing both HSPs would replicate better. Nevertheless, the results indicate that MV-HSP72 does not replicate faster than MV-NSE, and consequently the differences seen between MV and MV-HSP72 in the therapeutic trial cannot be attributed to a growth advantage of MV-HSP72.

Fig. 5. Increased HSP72 expression after infection with MV-HSP72. (a) Genome of MV-HSP72 depicted schematically: the nucleocapsid, phosphoprotein, matrix, fusion, haemagglutinin and polymerase proteins of the virus are represented, respectively, by N, P, M, F, H and L. The HSP72 gene was inserted between the H and L genes. (b) Detection of HSP72 by Western blotting: top row, HSP72 expression in Vero cells after infection with MV-NSE, MV-HSP72 and uninfected cells; middle row, detection of recombinantly expressed HSP72 by detection of the VSV tag inserted within the HSP72 gene; bottom row, detection of GAPDH. Vero cells infected with MV-HSP72 expressed twofold more HSP72 than uninfected or MV-NSE-infected cells. (c) Cell-free virus and (d) cell-associated growth curves of MV-NSE and MV-HSP72. No difference is seen in viral progeny between MV-NSE and MV-HSP72 (P=0.3, generalized linear model).
In conclusion, oncolytic viral therapy appears to be a promising new approach for the treatment of ATL. This study demonstrates that MV is effective in destroying human ATL cells in immunocompromised mice, and prolongs their survival by destroying relapsing leukemic cells.

**METHODS**

**Animals.** Female immunodeficient NOD/SCID mice (NOD.CB17-Prkdcscid/J)(Shultz et al., 1995) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and inoculated with $2 \times 10^6$ MET-1 leukemic cells intraperitoneally. Animals were monitored and weighed regularly after inoculation of tumour cells and during treatments until euthanasia. Blood was collected biweekly in animals with small tumour burdens, and weekly in animals with large tumour burdens. Mice were sacrificed by using CO$_2$ inhalation as soon as they developed signs of morbidity (>20% body-weight loss). During necropsy, tissues were collected for histopathology. All animal experiments were approved by the Institutional Animal Care and Use Committee of The Ohio State University.

**Cell lines.** MT-2 cells (Yamamoto et al., 1982) and Jurkat cells (Weiss et al., 1984) were maintained in Advanced RPMI 1640 medium (Invitrogen) supplemented with 10% FCS. MET-1 cells (Phillips et al., 2000) were derived from a patient with ATL and expanded in NOD/SCID mice. Vero cells (African green monkey, ATCC CCL-81) were grown in Advanced MEM medium (Invitrogen) supplemented with 10% FCS.

**MV.** MV strain Ed-NSE (Singh & Billeter, 1999) (MV-NSE) was grown and titrated on Vero cells by plaque forming assay. Briefly, serial tenfold dilutions of virus stock were incubated with Vero cells in six-well plates. After incubating the cells for 1 h with the viral dilutions, agar was overlayed onto the cell layer. Individual plaques (cytopathic effect) were counted 6 days post-infection after staining cells with neutral red. The amount of virus in the stock (titre) was expressed as the number of particles capable of forming plaques (p.f.u.) per unit volume (ml).

**Recombinant MV expressing HSP72 (MV-HSP72).** The HSP72 gene sequence was PCR amplified from the pT7–HSP72–VSV (Zhang et al., 2002b) vector (obtained from Dr M. Oglesbee) with Taq and Pfu (proofreader) polymerases using the sense (5'-TATATAACGGCG-TATAATGGCTTACACCAGACATCG-3') and antisense (5'-AATTAC-TAGTATGATCCGCAATCTTGGGGAAGG-3') primers that contain MluI and SpeI restriction sites, respectively. For detection purposes, the HSP72 gene has a VSV epitope tag (YTIDIEMLRGLK) inserted downstream of the start codon of HSP72. The 2138 bp ampiclon was inserted into a full-length infectious molecular clone of Edm-NSE (Singh & Billeter, 1999) by using the restriction enzymes MluI and SpeI (obtained from M. Billeter, Institute of Molecular Biology, University of Zurich, Switzerland) and obeying the reported paramyxovirus 'rule of six' requirement (Radecke et al., 1995). The integrity of the construct was confirmed by DNA sequencing. The virus was recovered according to a previously published protocol (Radecke et al., 1995) and viral stocks propagated in Vero cells and titres determined by plaque-forming assay.

To determine and compare the growth kinetics of MV-HSP72 and MV-NSE, Vero cells cultured in six-well plates were infected at an m.o.i. of 0.02 for 1 h at 37 °C. Every 12 h post-infection, cell lysates and supernatant were collected for cell-associated virus and progeny release curves, respectively. Serial tenfold dilutions of virus containing cell lysates or supernatant were assessed for the presence and levels of infectious virus in a 48-well microassay using Vero cells with neutral red. The amount of virus in the stock (titre) was expressed as the number of particles capable of forming plaques (p.f.u.) per unit volume (ml).

**ELISA for the detection of sIL-2Rα, TNF-α and IL-6.** ELISA reagents for measurement of sIL-2Rα concentrations were purchased from R&D Systems (recombinant human IL-2Rα, cat. no. 223-2A; anti-human IL-2Rα mouse mAb, clone 24204, Mouse IgG1; anti-human IL-2Rα biotinylated affinity-purified polyclonal goat antibody, Goat IgG). Human IL-6, mouse IL-6 and mouse TNF-α ELISA kits were acquired from R&D Systems (human IL-6 Quantikine ELISA kit; mouse IL-6 Quantikine ELISA kit; mouse TNF-α Quantikine ELISA kit).
Quantitative PCR for MV nucleocapsid RNA. Spleen samples were lysed and homogenized, and total RNA was extracted using an RNeasy kit (Qiagen). First strand cDNAs were synthesized using M-MLV reverse transcriptase and oligo(dT)$_{12-18}$ Primers as described by the manufacturer (Invitrogen), using 2 µg of RNA per reaction. Primers for the nucleocapsid of MV (Plumet & Gerlier, 2005) (sense 5'-AGTGAGAATGAGCTACCG-3' and antisense 5'-TGTCAGGGGTGTCGCC-3') and GAPDH (Li & Green, 2007) (sense 5'-CATCAATGACCCCTCATGAC-3' and antisense 5'-CAGGCCCACTTGATTGGA-3') were obtained for detection and quantification of the respective mRNA species. Plasmid clones for nucleocapsid and GAPDH were obtained (Plumet & Gerlier, 2005) and amplified, and tenfold serial dilutions of each plasmid were made ranging from 1 to 10$^6$ copies. Standard dilutions were stored in aliquots for single use at −20°C. Quantitative real-time PCR of nucleocapsid and GAPDH (corresponding to 0.5 µg of RNA per sample) was performed using a LightCycler 480 SYBR Green 1 Master kit (Roche Diagnostics) according to the manufacturer’s protocol in a LightCycler apparatus (Roche Diagnostics). Detection of nucleocapsid mRNA was used to assess MV infection. Quantitation of GAPDH was used for normalization.

Comparison of cell viability after infection with MV and MV-HSP72. Aliquots of 10$^6$ MT-2 cells were seeded in 12-well culture plates and incubated with MV, MV-HSP72 or mock treated (control) for 2 h at m.o.i. of 0.1. After 2 h of incubation, virus was washed and cells were incubated with Advanced RPMI supplemented with 10% FCS, at 37°C and in a 5% CO$_2$ atmosphere. At 24, 48 and 72 h post-infection, viable cells were counted by the trypan blue exclusion method in the MV, MV-HSP72 and control wells. The assay was performed in triplicate and repeated three times.

Flow cytometry analysis for the detection of HSP72. To determine HSP72 expression on the cell surface, Jurkat cells were incubated with MV or MV-HSP72, or were mock treated (control) at an m.o.i. of 0.1 for 2 h. After the end of the incubation period, cells were washed and cells maintained in Advanced RPMI/10% FCS. At 48 h post-infection cells were incubated with an antibody specific for HSP72 (clone C92F3A-5), or an isotype control (clone MOPC-21), conjugated to DyLight 488 from Assay Designs. Surface fluorescence was analysed with a BD FACScalibur flow cytometer (BD Biosciences).

Histopathology and immunohistochemistry. Tissues were fixed in 10% paraformaldehyde, paraffin embedded and stained with haematoxylin and eosin, or antibody specific for human Ki67 (clone K7-55; Dako) or a rabbit serum specific for MV nucleocapsid (Novus Biologicals). Immunostaining was done according to standard procedures using a biotin blocking kit and a target retrieval solution, both from Dako, and biotinylated secondary antibodies (Vector Laboratories). MET-1 cells have a perivascular pattern of infiltration, and tumour burden in tissues was graded according to the mean number of leukaemic cells around vessels in ten different high power fields at ×400 magnification. Consequently, neoplastic infiltration by tumour cells was considered minimal if the mean number of cells around vessels was fewer than five cells, mild if the mean number of cells was between 5 and 10 cells, moderate if the mean number of cells was between 10 and 15 cells, and marked if the number of cells was greater than 15 cells.

Statistical analysis. A mixed linear-regression model was used to compare control and MV-treated groups in the low-and high-tumour-burden therapeutic experiments over a post-inoculation time course (Figs 1 and 2). A two-sided, two-sample t-test was used to compare the mean concentration of mIL-6 between control and MV-NSE-treated groups on week 9 (Fig. 4) and to compare surface expression of HSP72 on Jurkat cells infected with MV-NSE and MV-HSP72 (Fig. 6b). A generalized linear model was used to study the differences in viral progeny evaluated by TCID$_{50}$ between MV-NSE and MV-HSP72 (Fig. 6c). A linear mixed model was used to compare the mean concentration of mIL-6 between control and MV-NSE-treated groups on week 9 (Fig. 4) and to compare surface expression of HSP72 on Jurkat cells infected with MV-NSE and MV-HSP72 (Fig. 6b). A generalized linear model was used to study the differences in viral progeny evaluated by TCID$_{50}$ between MV-NSE and MV-HSP72 (Fig. 6c). A linear mixed model was used to compare MT-2 cell counts between controls and cells infected with MV-NSE or MV-HSP72. Pairwise comparisons were performed and the P values are shown in Fig. 6(a). Survival times (Kaplan–Meier curves) of the MV-HSP72, MV and control groups were compared by using the log-rank test (Fig. 7).

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