genome were described previously [32]. Viral titers were calculated on the basis of the number of plaques on RK13 cells.

*2.2. Construction of m8Δ Expressing SCTs of Rat MHC-I.* The expression vectors, pEF/RT1AlSCTax180L and pEF/RT1AlSCNLEnv371L, which encode SCTs of rat MHC-I with Tax180–188 (Tax180) or human immunodeficiency virus type 1 (HIV-1) NL43 Env371–379 (NLEnv371) epitopes, respectively, were previously constructed [29]. To generate m8Δ expressing SCTs, peptide-$\beta_2$m-RT1A$^1$ fusion sequence in pEF/RT1AlSCTax180L or pEF/RT1AlSCNLEnv371L was amplified by PCR to add CpoI and FseI sites at the 5′ and 3′ end of the fusion constructs, respectively, and were ligated into the LC16m8ΔVNC110 genome that had been digested with CpoI and FseI. The ligated DNA was transfected into BHK cells that were infected with canarypox virus, as described previously [32]. The recombinants were selected by plaque ELISA using an anti-rat MHC-I antibody (clone OX-18; BD PharMingen Co., San Diego, CA) and were then subjected to Western blotting to confirm the proper protein expression.

*2.3. Protein Analysis.* For plaque ELISA, recombinant VVs were infected to RK13 cells on 6 well plates at approximately 100 pfu/well. After incubation for 72 h at 33°C, the infected cells were fixed with 2% paraformaldehyde solution followed by permeabilization with 0.5% NP40 for 1 min. The fixed cells were blocked with 5% skim milk in PBS for 30 min and incubated with an anti-rat MHC-I antibody (clone OX-18; BD PharMingen Co.) followed by incubation with an alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma-Aldrich). After staining with alkaline phosphatase substrates, the plaques with dark blue color were collected as positive clones.

For Western blotting, cells were resuspended in ice-cold extraction buffer (20 mM HEPES [pH 7.9], 10 mM KCl, 1 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF, 1 $\mu$g/mL aprotinin, and 1 $\mu$g/mL leupeptin) and gently rocked for 30 minutes. After centrifugation at 14,000 ×g for 20 minutes at 4°C, the supernatant was collected as a whole cell extract. The protein concentration of each sample was determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Fifty $\mu$g of whole cell extracts was separated by 8% SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with an anti-rat MHC-I antibody and then with an anti-mouse Ig antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). Antibodies bound to the filter were detected by the enhanced chemiluminescence method (Amersham).

*2.4. A Flow-Cytometric CTL Killing Assay.* EGFP-expressing target cells (2.5–5.0 × $10^4$ cells/well) were cocultured with CTLs (2.5 × $10^5$–1 × $10^6$ cells/well). These mixed cultures were immediately subjected to flow-cytometric analysis or were incubated for indicated days and then subjected to flow-cytometric analysis. Cytofluorometry was done on a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed with Cell Quest software. Target cells were clearly gated away from CTLs by light-scatter properties and EGFP expression.

*2.5. IFN-γ Production Assay.* The 4O1/C8 (1 × $10^5$/well) was mixed with various stimulator cells (2 × $10^4$/well). After indicated period of mixed culture, supernatants were harvested and subjected to rat IFN-γ ELISA (eBioscience Inc., San Diego, CA) in accordance with the manufacturer's instructions.

*2.6. Cell Viability Assay.* Cells were infected with VVs and then incubated for indicated periods. In some experiments, cells were stimulated with formalin-fixed FPM1 cells for 2 days and then infected with VVs. The number of growing cells was determined by using a cell counting kit-8 (Dojinndo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. Cell viabilities are expressed as percentages of cell survival of mock-infected cultures, as described previously [24].

*2.7. Statistical Analysis.* Comparisons between individual data points were made using a Student's $t$-test. Two-sided $P$ values <0.05 were considered statistically significant.

## 3. Results

*3.1. Rat HTLV-I-Infected Cells Were Susceptible to the Killing by Attenuated Vaccinia Strain, m8Δ.* To develop a safe and effective smallpox vaccine and vector virus, we have previously constructed genetically stable m8Δ, which is less pathogenic than its parental mO due to the deletion of B5R gene [22], and successfully applied it for animal studies of HIV-1 vaccine developments [32–34]. In this study, to determine whether m8Δ possesses cytolytic activity against HTLV-I-infected cells, a rat HTLV-I-infected cell line, FPM1 was infected with m8Δ or mO. As shown in Figure 1(a), we observed the gradual reduction of cell viability in FPM1 cells infected with m8Δ at multiplicity of infection (MOI) 0.1 and confirmed the significant difference in cell viability between m8Δ - and mock-infected cells at 4 days after infection, indicating the induction of cytolysis of FPM1 cells by m8Δ. The cytolysis induction by mO was more efficient than that by m8Δ at MOI 0.1, because significant difference in cell viability was observed after 2 days of infection. Similar levels of significant cytolysis induction were observed in FPM1 cells infected with either m8Δ or mO at MOI 0.5. We next examined the virus replication in the cells infected with VV at MOI 0.1 and confirmed 2.1 × $10^3$ and 1.2 × $10^4$ fold increase of infectious m8Δ and mO, respectively, at 3 days after infection (Figure 1(b)). These results indicate that the attenuated m8Δ possesses lower level of oncolytic activity compared with its parental mO and that increasing the virus inoculum can compensate the reduced activity. In addition, we have maintained the virus-infected cells for extended periods and confirmed that all the cells used in Figure 1(a) were eventually killed by VVs (data not shown). Thus, prolonged cultivation could also improve the efficacy of oncolysis by highly attenuated VVs.

*3.2. A Tax-Specific CTL Line, 4O1/C8, Was Resistant to Killing by m8Δ.* Virus-specific CTLs that play important roles in

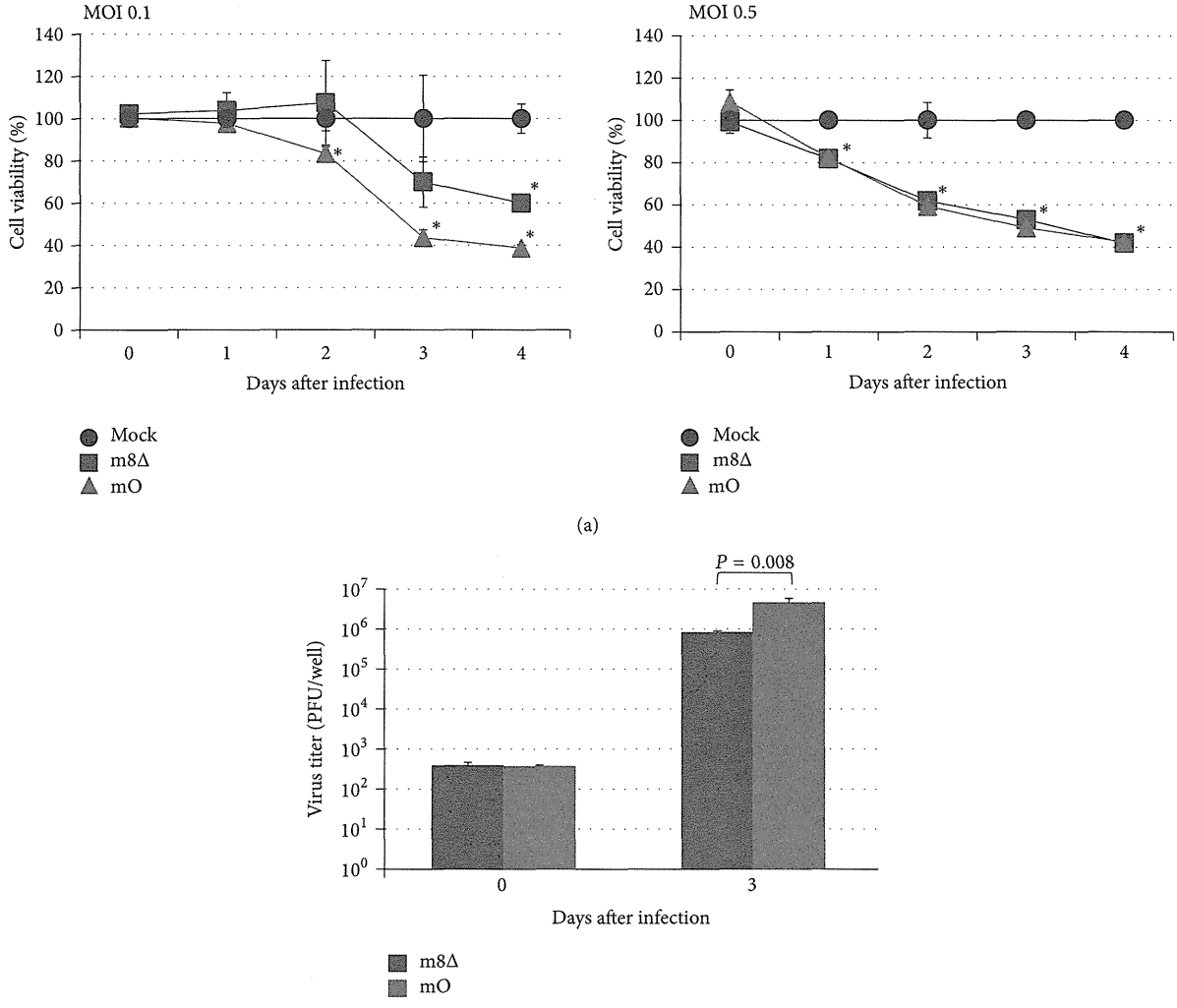



FIGURE 1: Viability of FPM1 cells infected with attenuated VVs. (a) FPM1 cells were exposed to m8Δ (■), mO (▲), or PBS (●) at indicated MOI for 2 hrs. After extensive wash, the cells were cultured for indicated periods and the cell growth was assessed by using cell counting kit 8. The cell viabilities are expressed as percentages of the cell survival of mock-infected cultures. The data are presented as mean ± SD of triplicate wells. Asterisks indicate statistical significance ($P < 0.05$) compared to the mock-infected controls. (b) The proliferation of VVs in FPM1 cells infected with the virus at MOI 0.1 was determined by titrating the cell lysates collected at indicated days. The data are presented as mean ± SD of triplicate wells. Statistical significance was determined as $P < 0.05$. Similar results were obtained in two independent experiments.

eradication of virus-infected cells should not be eliminated by oncolytic viruses during treatment. Thus, it is important to confirm the resistance of CTLs to cytolysis by m8Δ. To assess the susceptibility of Tax-specific CTLs to killing by m8Δ, 4O1/C8 cells were stimulated with formalin-fixed FPM1 cells to induce cell proliferation and then were exposed to m8Δ at MOI 2. As shown in Figure 2(a), exposure of 4O1/C8 to m8Δ did not influence the growth of the CTLs. In contrast, dramatic decrease in the viability of FPM1 was observed after infection of m8Δ at MOI 2 (Figure 2(b)). The enhanced cytolysis of FPM1 should be due to higher amount of inoculated virus compared with that used in Figure 1. The assessment of virus titer in the virus-exposed 4O1/C8 demonstrated that the titer of 4O1/C8-associated virus was stable during the first 4 days, suggesting that m8Δ was not able to proliferate in the CTLs but was stable for several days in the presence of the CTLs (Figure 2(c)). Alternatively, it is also possible that low levels of m8Δ proliferation may compensate the natural reduction of the virus titer in the culture. These results indicated that 4O1/C8 is resistant to the cytolysis by m8Δ and suggested that virotherapy using m8Δ does not affect the function of CTLs. Thus, m8Δ could be applicable for the combination therapies using oncolytic viruses and antigen-specific T cells against HTLV-I-infected cells.

*3.3. Lack of IFN-γ Production Was Correlated with the Resistance of FPM1V.EFGFP/8R Cells to Killing by 4O1/C8 CTL.* We have previously established an assay system by which we can evaluate the susceptibility of HTLV-I-infected

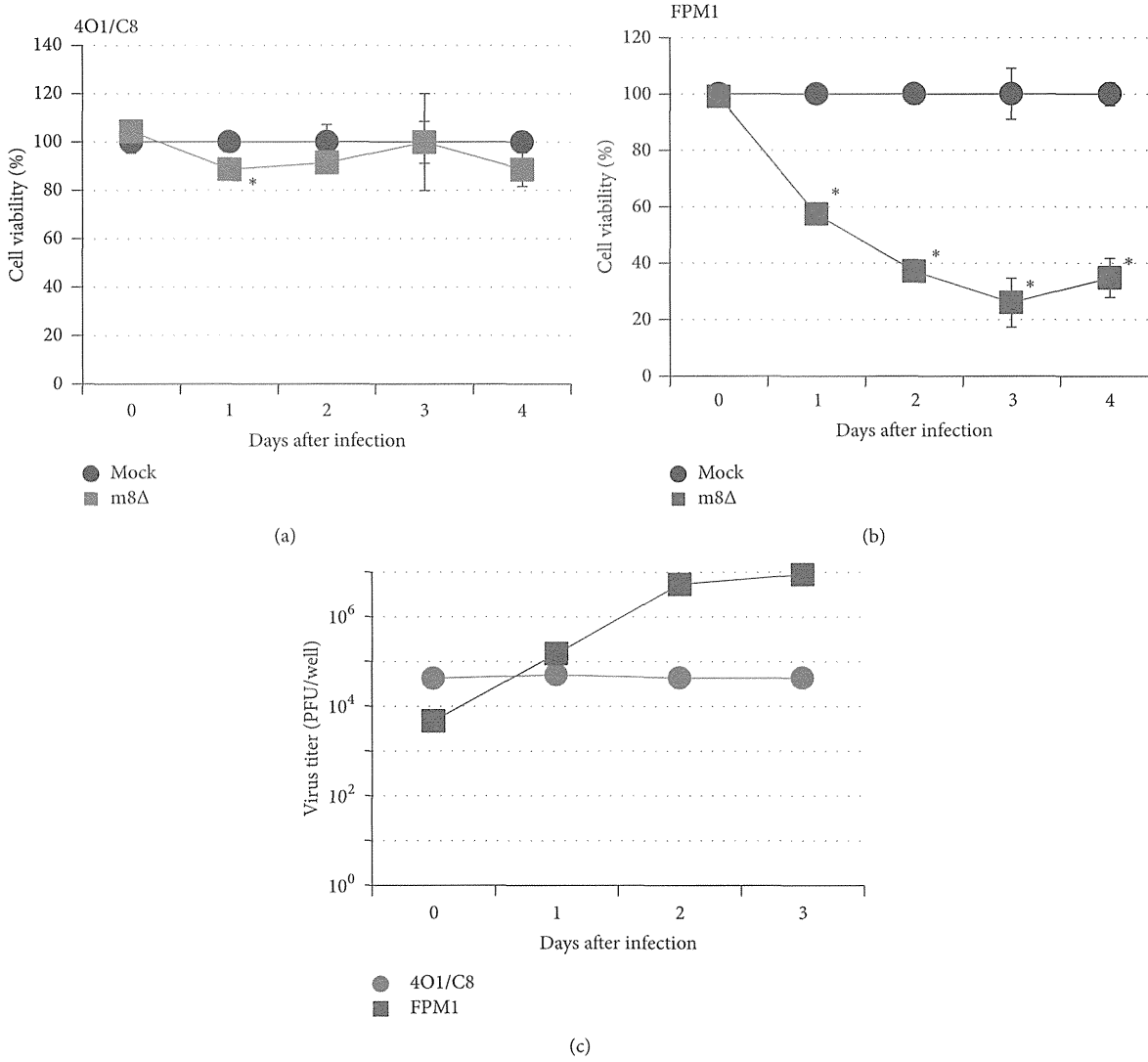



FIGURE 2: A Tax-specific CTL line, 4O1/C8, was resistant to killing by m8Δ. The 4O1/C8 (a) or FPM1 (b) cells were exposed to m8Δ (■) or PBS (●) at MOI 2 for 2 hrs. After extensive wash, the cells were cultured for indicated periods and the cell growth was assessed by using cell counting kit 8. The cell viabilities are expressed as percentages of the cell survival of mock-infected cultures. The data are presented as mean ± SD of triplicate wells. Asterisks indicate statistical significance ($P < 0.05$) compared to the mock-infected controls. Similar results were obtained in two independent experiments. (c) The proliferation of VVs in 4O1/C8 (●) or FPM1 (■) cells infected with the virus at MOI 2 was determined by titrating the cell lysates collected at indicated days. The data are presented as mean of duplicate wells.

cells to CTL killing by flow-cytometric analysis [25]. In this study, we used EGFP-expressing subclones of FPM1 cells, FPM1V.EFGFP and FPM1V.EFGFP/8R, as target cells of Tax-specific CTLs. FPM1V.EFGFP/8R cells were previously isolated by continuously cocultivating with 4O1/C8 cells and were shown to be resistant to killing by 4O1/C8 due to downregulation of MHC-I but not Tax expression [25]. As we have previously reported, mixed culture of FPM1V.EFGFP and 4O1/C8 cells resulted in the dramatic decrease of EGFP-positive FPM1V.EFGFP fractions (Figure 3(a)). In contrast, the percentage of FPM1V.EFGFP/8R increased in 4 days of mixed culture with 4O1/C8 cells, indicating the resistance of FPM1V.EFGFP/8R to killing by 4O1/C8. To determine whether the activation of 4O1/C8 was induced in the mixed culture, IFN-$\gamma$ production in the supernatants was evaluated. As shown in Figure 3(b), mixed culture of 4O1/C8 cells with FPM1V.EFGFP induced IFN-$\gamma$ secretion whereas that with FPM1V.EFGFP/8R did not. Thus, production of IFN-$\gamma$ in the mixed culture correlated with killing of the HTLV-I-infected cells by 4O1/C8. We next infected the mixed culture of 4O1/C8 and FPM1V.EFGFP/8R cells with m8Δ to determine whether cytolysis of the CTL-resistant cells by the oncolytic virus induced the activation of the Tax-specific CTLs. As shown in Figure 3(a), slight decrease of GFP positive cell fraction (7.4 ± 0.2%) was observed in 4 days of mixed culture with m8Δ, which was in stark contrast to the apparent increase of GFP positive cell fraction in the absence of m8Δ (22.4 ± 0.3%), demonstrating that cytolysis

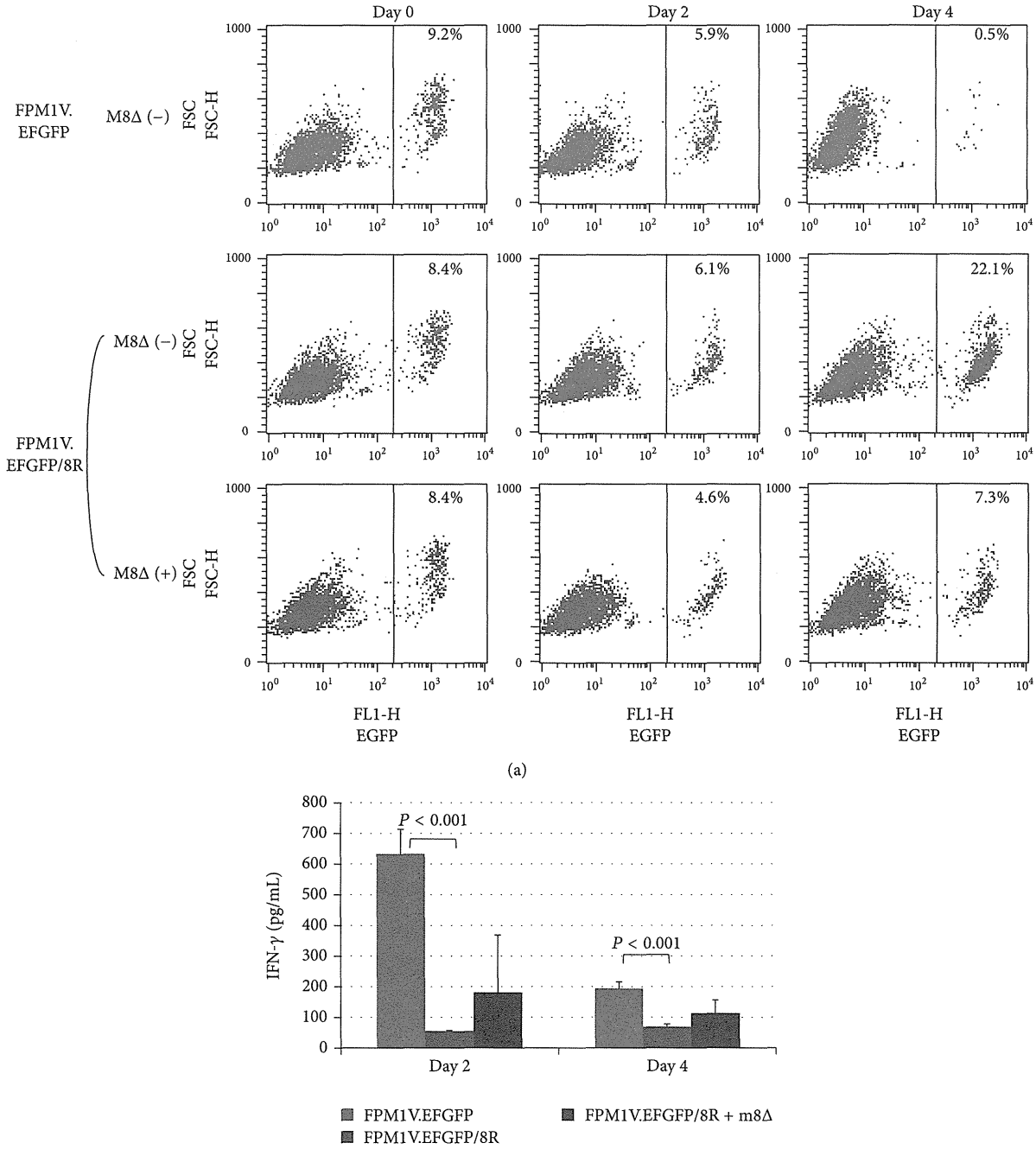



FIGURE 3: Lack of IFN-$\gamma$ production was correlated with the resistance of FPM1V.EFGFP/8R cells to killing by 4O1/C8. (a) FPM1V.EFGFP or FPM1V.EFGFP/8R cells ($5 \times 10^4$/well) were mixed with 4O1/C8 cells ($5 \times 10^5$/well) at an E : T ratio of 10 : 1 in the presence or absence of m8Δ ($1 \times 10^5$ PFU/well) and subjected to flow-cytometric analysis for the expression of EGFP at the indicated days. Percentage of EGFP positive cells is indicated in each panel. (b) Production of IFN-$\gamma$ in the supernatants of mixed culture prepared in (a) was measured by ELISA at the indicated days of culture. The data represent the mean ± SD of triplicate wells. Statistical significance was determined as $P < 0.001$. Similar results were obtained in two independent experiments.

of FPM1V.EFGFP/8R was induced by m8Δ. There were no cells surviving after extended cultivation of the virus-infected FPM1V.EFGFP/8R cells in the experiment (data not shown). However, IFN-$\gamma$ production was not detected in the mixed culture with m8Δ (Figure 3(b)), indicating that cytolysis of the HTLV-I-infected cells by m8Δ was independent of CTL activation.

*3.4. Characterization of Recombinant VVs Expressing SCTs of Rat MHC-I.* To improve the efficiency of oncolytic viruses, various types of modifications have been reported [17]. In this study, we have utilized SCTs with a Tax-epitope to enhance the oncolytic ability of m8Δ against HTLV-I-infected cells in combination with Tax-specific CTLs. Tax180 epitope was previously identified as an RT1.$A^l$-restricted CTL epitope recognized by a Tax-specific CTL line, 4O1/C8 [14]. As a negative control, we have chosen a putative RT1.$A^l$-restricted epitope in the envelope of HIV-1 NL4-3 strain, NLEnv371, which was determined to have the same point as Tax180 epitope scored by epitope prediction data via http://www.syfpeithi.de/ [35]. A schematic representation of SCTs is shown in Figure 4(a). We have introduced the coding sequence of SCTs with Tax180 or NLEnv371 into the genome of m8Δ and obtained m8Δ/RT1AlSCTax180L or m8Δ/RT1AlSCNLEnv371L, respectively. The SCT protein expression by m8Δ/RT1AlSCTax180L was examined in RK13 cells. Among the 4 clones tested, 2 clones (Numbers 7 and 8) appeared to express SCTs and clone Number 7 was used for further studies (Figure 4(b)). The expression of SCTs by m8Δ/RT1AlSCNLEnv371L was also confirmed by Western blotting (Figure 4(c)). We further assessed the function of the SCTs expressed by m8Δ/RT1AlSCTax180L, by infecting the virus to RK13 cells and coculturing the infected cells with 4O1/C8. As shown in Figure 4(d), RK13 cells infected with m8Δ/RT1AlSCTax180L were able to induce IFN-$\gamma$ secretion by 4O1/C8. In contrast, RK13 cells infected with m8Δ/RT1AlSCNLEnv371L induced little amount of IFN-$\gamma$ secretion by the Tax-specific CTLs. These results indicated that SCTs expressed by m8Δ/RT1AlSCTax180L were able to activate Tax-specific CTLs. Thus, it is expected that m8Δ/RT1AlSCTax180L possesses dual functions of both lysing HTLV-I-infected cells and activating Tax-specific CTLs.

*3.5. Combined Effects of 4O1/C8 and m8Δ on Killing of CTL-Resistant HTLV-I-Infected Cells.* To examine the combined effects of Tax-specific CTLs and m8Δ expressing SCTs, we next infected FPM1V.EFGFP/8R cells with m8Δ/RT1AlSCTax180L or m8Δ/RT1AlSCNLEnv371L and cocultivated the infected cells with 4O1/C8. As shown in Figure 5, the proportion of FPM1V.EFGFP/8R cells in the mixed culture clearly decreased at 4 days after m8Δ/RT1AlSCNLEnv371L infection compared to the mock-infected controls. The decrease of FPM1V.EFGFP/8R cells was MOI-dependent and may be due to oncolytic ability of the virus, since IFN-$\gamma$ production was not detected in the mixed culture (Figure 6). A greater reduction of EGFP-positive cells was observed in the mixed culture of FPM1V.EFGFP/8R cells infected with m8Δ/RT1AlSCTax180L. In particular, m8Δ/RT1AlSCTax180L infection at MOI 10 induced most dramatic elimination of FPM1V.EFGFP/8R cells. This may be due to the combined effects of oncolytic activity and activation of 4O1/C8 cells induced by SCTs with Tax presentation, since IFN-$\gamma$ production was clearly induced in the mixed culture (Figure 6). Induction of IFN-$\gamma$ was partly due to the direct effect of m8Δ/RT1AlSCTax180L to 4O1/C8, since direct exposure of 4O1/C8 to m8Δ/RT1AlSCTax180L, but not to m8Δ/RT1AlSCNLEnv371L, resulted in the production of IFN-$\gamma$ (data not shown). We also examined the cytolytic activity of m8Δs expressing SCTs in the absence of 4O1/C8 to determine whether expression of different epitopes affects the lysis of FPM1V.EFGFP/8R cells. As shown in Figure 7(a), equivalent levels of cell growth inhibition were observed in FPM1V.EFGFP/8R cells infected with either m8Δ/RT1AlSCTax180L or m8Δ/RT1AlSCNLEnv371L. These results demonstrated that there is no difference in direct cytolytic ability between m8Δ/RT1AlSCTax180L and m8Δ/RT1AlSCNLEnv371L and further indicated that significantly strong reduction of EGFP-positive cell fraction observed in the mixed culture of 4O1/C8 and m8Δ/RT1AlSCTax180L-infected FPM1V.EFGFP/8R cells was due to the additional cytotoxic activity of 4O1/C8 activated by m8Δ/RT1AlSCTax180L-mediated epitope presentation. Finally, we have evaluated the virus titers in FPM1V.EFGFP/8R cells infected with VVs in the presence or absence of 4O1/C8 cells to determine whether the CTLs influence the replication of VVs. As shown in Figure 7(b), we have not observed any significant differences of the virus titer between VV-infected FPM1V.EFGFP/8R cells cultivated with 4O1/C8 and those without 4O1/C8 during the first 4 days after infection regardless of the VVs used, although slight reduction of VV titer was induced by the addition of 4O1/C8 cells in most of the samples examined. Thus, the CTLs did not significantly affect the replication of VVs in the present experiments.

## 4. Discussion

The primary effect of oncolytic virotherapy depends on the vigorous viral replication and spread within tumor tissues. In addition, it has been reported that oncolytic virus-mediated tumor destruction leads to the activation of tumor-specific immune responses and the improved efficacy of virotherapy [36, 37]. Thus, activation of tumor-specific immune responses could be another strategy to enhance tumor specific killing by attenuated oncolytic viruses. Indeed, GM-CSF-encoding VV or herpes virus has been developed to effectively induce tumor regression [38, 39]. Encoding a tumor antigen within an oncolytic virus also enhanced the tumor-specific immune responses and the efficacy of tumor eradication [40, 41]. Based on these previous reports, we have developed a novel combination therapy against HTLV-I tumor in a rat model system, which consists of a Tax-specific CTL line and an attenuated VV expressing SCTs with a Tax-epitope. In line with the previous reports, our present results demonstrated the effective cytolysis of HTLV-I-infected cells by an attenuated VV and the synergistic effects between activated virus-specific T cells and oncolytic viruses toward eliminating

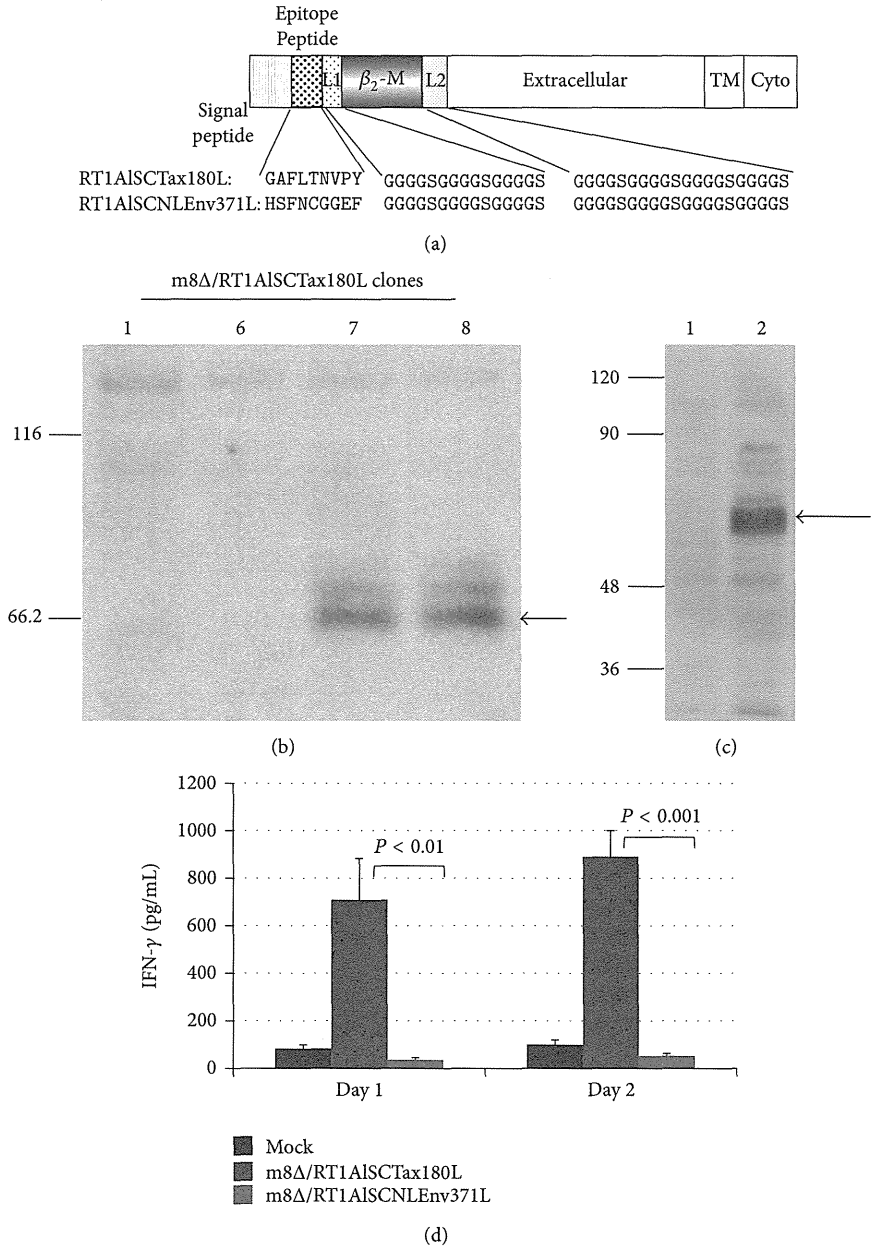
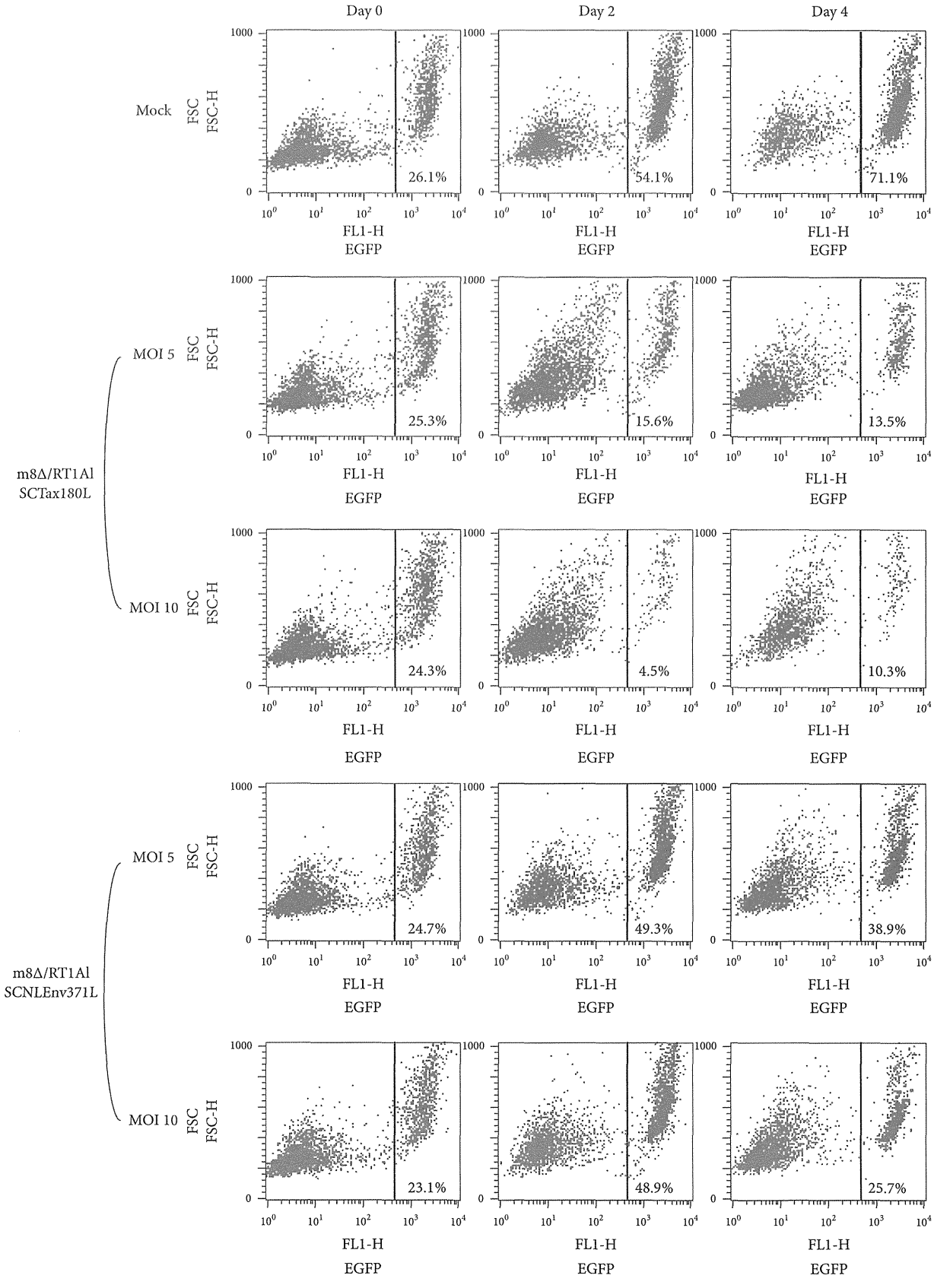


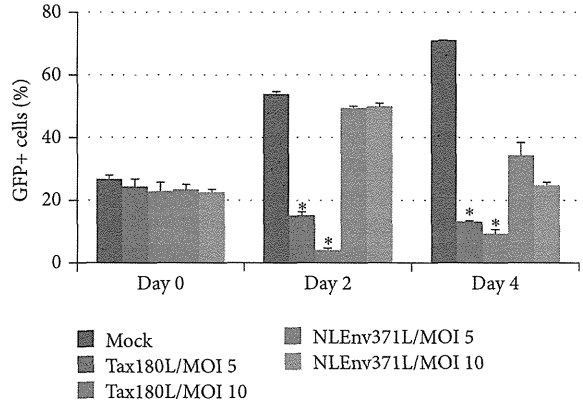

FIGURE 4: Characterization of m8Δ expressing SCTs. (a) Diagram of SCTs encoding Tax180–188 or NLEnv371–379 linked to $\beta_2$m and RT1.A$^l$ molecules with two linkers. L1, linker 1; L2, linker 2; TM, transmembrane region; Cyto, cytoplasmic region. ((b) and (c)) Whole cell extracts were isolated from RK13 cells infected with indicated clones of m8Δ/RT1AlSCTax180L (b) or RK13 cells infected with m8Δ (lane 1) or m8Δ/RT1AlSCNLEnv371L (lane 2) (c) and 50 $\mu$g of each lysate was subjected to Western blotting analysis for the expression of the SCT of MHC-I proteins. Arrows indicate the SCT of MHC-I proteins detected by an anti-rat MHC-I antibody. Molecular weight markers are indicated (kDa) on the left margin. (d) RK13 cells were exposed to m8Δ/RT1AlSCTax180L, m8Δ/RT1AlSCNLEnv371L, or PBS for 2 hrs. After extensive wash, the cells were cocultivated with 4O1/C8 for indicated days. Production of IFN-$\gamma$ in the supernatants was measured by ELISA. The data represent the mean ± the SD of triplicate wells. Statistical significance was determined as $P < 0.01$. Similar results were obtained in two independent experiments.

tumor cells. Introduction of SCT system should be extremely important in the case of HTLV-I infection, because repression of Tax expression is frequently observed in HTLV-I carriers and is a possible cause of the declined virus-specific immune responses [8]. By using SCTs, effective presentation of Tax-epitopes could be expected to induce proper activation of anti-HTLV-I T cell responses, even when Tax expression is repressed. Since repression of antigen presentation by MHC-I represents a common feature of tumor cells and is one of the mechanisms of tumor immune evasion, encoding a SCT of MHC-I gene within an attenuated oncolytic virus could also be effective against a broad range of tumors. Moreover,




(a)

Figure 5: Continued.




(b)

FIGURE 5: Combined effects of 4O1/C8 and m8Δ on killing of CTL-resistant HTLV-I-infected cells. (a) FPM1V.EFGFP/8R cells were exposed to m8Δ/RT1AlSCTax180L, m8Δ/RT1AlSCNLEnv371L, or PBS for 2 hrs at indicated MOI. After extensive wash, the cells were cocultivated with 4O1/C8 for indicated periods and subjected to flow-cytometric analysis for the expression of EGFP at the indicated days. Percentage of EGFP positive cells is indicated in each panel. (b) Bar graph of the flow-cytometric data. The data are presented as mean ± SD of triplicate wells. Asterisks indicate statistical significance ($P < 0.01$) compared to the m8Δ/RT1AlSCNLEnv371L-infected cells with corresponding MOI.

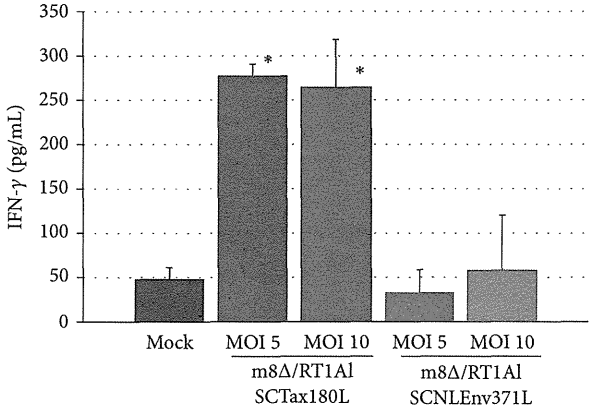



FIGURE 6: Production of IFN-γ by 4O1/C8 that was cocultured with recombinant m8Δ-exposed FPM1V.EFGFP/8R cells. FPM1V.EFGFP/8R cells were exposed to m8Δ/RT1AlSCTax180L, m8Δ/RT1AlSCNLEnv371L, or PBS for 2 hrs at indicated MOI. After extensive wash, the cells were cocultivated with 4O1/C8 for 2 days. Production of IFN-γ in the supernatants of mixed culture was measured by ELISA. The data represent the mean ± SD of triplicate wells. Asterisks indicate statistical significance ($P < 0.05$) compared to the mock-infected controls.

it has been reported that combining adoptive cell therapy with oncolytic viruses leads to effective elimination of tumor cells [42]. In addition to the primary effects of direct killing of tumor cells, tumor-specific T cells were also known to enhance oncolysis in vivo by carrying oncolytic viruses to distal tumor sites [43]. Thus, combined administration of SCT-expressing oncolytic virus and tumor-specific T cells could be one of the ideal combinations to maximize antitumor effects. However, it is also important to note that CTLs may possibly have inhibitory effects on the replication of oncolytic viruses. Although significant inhibition of the virus replication was not induced by 4O1/C8 cells as shown in Figure 7(b), IFN-γ produced by the CTLs could have the potential to exert adverse effects on the virus replication in different conditions. Thus, it should be important to consider the effects of CTLs on virus replication and oncolytic activity when we design the combination therapy of antigen-expressing oncolytic viruses and tumor-specific T cells.

VVs are known to have unique biological properties, including resistance to antibody- and compliment-mediated neutralization abilities in blood [44, 45]. Taking advantage of the properties, a recent clinical trial has successfully demonstrated the intravenous delivery and replication of oncolytic VVs in metastasized tumor tissue [21]. Since HTLV-I primarily infects T cells in the blood and expands through the bloodstream, it is reasonable to assume that VVs could also reach systemically spreading HTLV-I-infected cells after intravenous administration. In addition, the unique biological nature of VVs made them possible to spread systemically even in patients with a history of live VV vaccination [21]. Thus, it is anticipated that oncolytic VVs could be effective in HTLV-I-infected individuals with anti-VV immunity.

The efficacy of a cancer treatment has to be balanced against its potential toxicity to normal cells. The safety of m8Δ has been demonstrated through the use of its natural counterpart, m8, for smallpox vaccine [23]. It is of note that m8Δ infection showed neither viral replication in nor cytolysis of Tax-specific CTLs (Figure 2), further indicating the reduced toxicity to normal cells. However, due to the lack of *B5R* gene, oncolytic activity of m8Δ was shown to be declined [24]. Our present observation also confirmed the weaker oncolytic activity of m8Δ than that of mO in a rat HTLV-I-infected cell line (Figure 1). In addition, we observed the lack of Tax-specific T cell activation in the mixed culture of FPM1V.EFGFP/8R cells infected with m8Δ (Figure 3) or m8Δ/RT1AlSCNLEnv371L (Figure 6). Since FPM1V.EFGFP/8R cells are known to express Tax protein [25], it is anticipated that cytolysis of FPM1V.EFGFP/8R by m8Δ should lead to the release of the viral antigen and

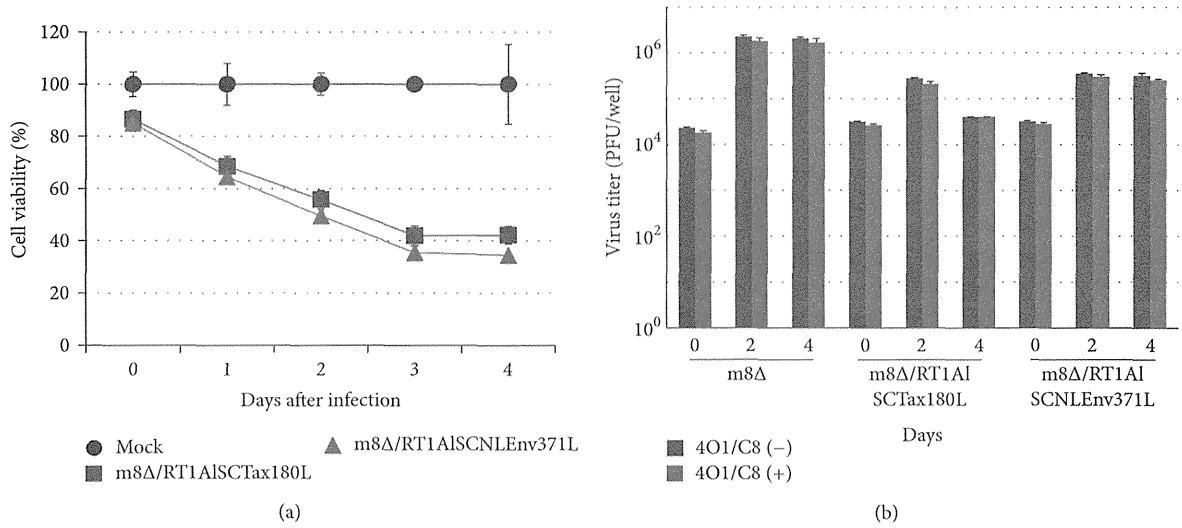



FIGURE 7: Viability of and viral replication in FPM1V.EFGFP/8R cells exposed to recombinant m8Δs. (a) FPM1V.EFGFP/8R cells were exposed to m8Δ/RT1AlSCTax180L (■), m8Δ/RT1AlSCNLEnv371L (▲), or PBS (●) for 2 hrs at MOI 5. After extensive wash, the cells were cultured for indicated periods and the cell growth was assessed by using cell counting kit 8. The cell viabilities are expressed as percentages of the cell survival of mock-infected cultures. The data are presented as mean ± SD of triplicate wells. (b) FPM1V.EFGFP/8R cells ($5 \times 10^4$/well) were exposed to m8Δ at MOI 2, or m8Δ/RT1AlSCTax180L, or m8Δ/RT1AlSCNLEnv371L at MOI 5. After extensive wash, the cells were cultured in the presence or absence of 4O1C8 cells ($2.5 \times 10^5$/well) for indicated periods and were collected for the evaluation of virus proliferation. The proliferation of VVs was determined by titrating the cell lysates. The data are presented as mean ± SD of triplicate wells.

the activation of 4O1/C8 cells. Indeed, our previous results showed that addition of Tax180 peptide directly to the culture led to the activation of 4O1/C8 [25]. Thus, it is possible that cytolysis of FPM1V.EFGFP/8R cells by m8Δ was slowly processed even when m8Δ efficiently replicated in the cells and that the amount of Tax protein released from destructed cells was not enough to activate Tax-specific CTLs in the present experimental condition. Under this condition, to compensate the reduced oncolytic ability of m8Δ, we introduced a gene encoding SCT with Tax180 within the genome of m8Δ and showed the improved cytolysis of FPM1 cells by Tax-specific CTLs without altering direct oncolytic ability of m8Δ. It seemed that the ability to induce T cell activation by m8Δ/RT1AlSCTax180L could be fairly strong, because rabbit RK13 cells also became capable of inducing IFN-$\gamma$ production by 4O1/C8 cells after m8Δ/RT1AlSCTax180L infection (Figure 4(d)). Since uncontrolled activation of T cells may result in normal cell injury, it is also necessary to carefully evaluate the safety of the virus in vivo. In addition, there are other strategies to overcome the reduced oncolytic activity of attenuated viruses [17]. As for m8Δ, Hikichi et al. have successfully developed a microRNA-regulated system, by which m8Δ can selectively express B5R in tumor cells and demonstrated full restoration of its oncolytic activity [24]. These strategies could be also combined to further enhance oncolytic activities without damaging normal cells.

ATL is known to acquire resistance to conventional chemotherapy and has a poor prognosis. Although allo-HSCT had been developed for the treatment of ATL, patients who are eligible for the treatment are still limited [46, 47]. Recently, a novel promising therapy using a humanized anti-CCR4 monoclonal antibody has been reported to be effective against ATL [48]. In addition, there are other novel target proteins discovered for T cell therapies against ATL, including NY-ESO-1 [49], and hTERT [50]. Since the VV encoding SCTs developed in this study as well as previously reported VSV [19] have unique mechanisms of action against HTLV-I-infected cells, it is possible that combination of these oncolytic viruses with the recently developed immune therapies could further enhance the efficacy of ATL treatment.

## 5. Conclusion

We demonstrated that an attenuated VV, m8Δ, possesses oncolytic activity to HTLV-I-infected cells and that m8Δ encoding SCT with a Tax-epitope enhances the cytolysis of CTL-resistant HTLV-I-infected cells in combination with Tax-specific CTLs. This newly established VV expressing SCT should have combining tumor debulking activity of direct tumor lysis and activation of tumor specific CTLs. The combination of epitope specific-CTLs and attenuated VVs encoding SCTs with corresponding epitopes could be effective tool to eradicate tumors escaping from immune surveillance.

## Abbreviations

ATL: Adult T cell leukemia
allo-HSCT: Allogeneic hematopoietic stem cell transplantation
$\beta_2$m: $\beta_2$-microgrobulin
CTL: Cytotoxic T lymphocyte
HIV-1: Human immunodeficiency virus type 1

HTLV-I: Human T cell leukemia virus type I
HAM/TSP: HTLV-I-associated myelopathy/tropical spastic paraparesis
MOI: Multiplicity of infection
MHC-I: Major histocompatibility complex class I
SCT: Single chain trimer
VV: Vaccinia virus
VSV: Vesicular stomatitis virus.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review*

# Vaccinia Virus LC16m8Δ as a Vaccine Vector for Clinical Applications

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**Abstract:** The LC16m8 strain of vaccinia virus, the active ingredient in the Japanese smallpox vaccine, was derived from the Lister/Elstree strain. LC16m8 is replication-competent and has been administered to over 100,000 infants and 3,000 adults with no serious adverse reactions. Despite this outstanding safety profile, the occurrence of spontaneously-generated large plaque-forming virulent LC16m8 revertants following passage in cell culture is a major drawback. We identified the gene responsible for the reversion and deleted the gene (*B5R*) from LC16m8 to derive LC16m8Δ. LC16m8Δ is non-pathogenic in immunodeficient severe combined immunodeficiency (SCID) mice, genetically-stable and does not reverse to a large-plaque phenotype upon passage in cell culture, even under conditions in which most LC16m8 populations are replaced by revertants. Moreover, LC16m8Δ is >500-fold more effective than the non-replicating vaccinia virus (VV), Modified Vaccinia Ankara (MVA), at inducing murine immune responses against pathogenic VV. LC16m8Δ, which expresses the SIV *gag* gene, also induced anti-Gag $CD8^+$ T-cells more efficiently than MVA and another non-replicating VV, Dairen I minute-pock variants (DIs). Moreover, LC16m8Δ expressing HIV-1 Env in combination with a Sendai virus vector induced the production of anti-Env antibodies and $CD8^+$ T-cells. Thus, the safety and efficacy of LC16m8Δ mean that it represents an outstanding platform for the development of human vaccine vectors.

**Keywords:** LC16m8Δ; LC16m8; vaccinia virus; reversion; *B5R*; MVA; DIs; SIV; HIV

## 1. Introduction

### *1.1. First-Generation Smallpox Vaccines*

Smallpox was eradicated worldwide in the 1970s [1,2]. However, serious public health concerns due to the threat of bioterrorism [3] and natural outbreaks of monkeypox [4,5] at the start of the 21st century have highlighted the necessity for a vaccinia virus (VV)-based smallpox vaccine. Existing vaccine stockpiles have not been updated since the 1970s; because these early vaccines are lymph-derived vaccines produced by propagating vaccine viruses in the skin of animals (*i.e.*, first-generation vaccines (Table 1)), they do not meet good manufacturing practice (GMP) standards [6–8]. Therefore, they are at risk for adventitious microbial contamination. Moreover, these vaccines occasionally caused serious adverse effects (e.g., progressive vaccinia, eczema vaccinatum and post-vaccinial encephalitis) due to the pathogenicity of the vaccine viruses used [9,10].

**Table 1.** Smallpox vaccines and candidate vaccines classified according to generation.

| Generation | Product | Platform | Parental Strain |
|---|---|---|---|
| First-generation | Lister/Elstree | Lymph-derived | Lister/Elstree |
| | Dryvax | Lymph-derived | NYCBH [a] |
| | Ikeda | Lymph-derived | Ikeda |
| | Dairen I | Lymph-derived | Dairen I |
| Second-generation | ACAM1000 | Clonal virus grown in MRC-5 cells | Dryvax |
| | ACAM2000 | Clonal virus grown in Vero cells | ACAM1000 |
| | Elstree-BN | Lister/Elstree lymph-derived virus passaged in CEF [b] | Lister/Elstree |
| | CCSV | NYCBH lymph-derived virus passaged in MRC-5 cells | NYCBH |
| Third-generation | LC16m8 [c] | Minute-pock-forming, temperature-sensitive variant virus | Lister/Elstree |
| | IMVAMUNE (MVA [d]) | MVA571 additionally passaged in CEF | MVA571 |
| | DIs [e] | Minute-pock-forming variant virus passaged in eggs | Dairen I |
| Fourth-generation | LC16m8Δ | Derived from LC16m8 by deleting the *B5R* gene | LC16m8 |
| | NYVAC | Attenuated clonal Copenhagen strain generated by deleting 18 non-essential genes | Copenhagen |

[a] New York City Board of Health; [b] chicken embryo fibroblast; [c] Lister Clone 16m8; [d] Modified Vaccinia Ankara; [e] Dairen I minute-pock variants.

### *1.2. Second-Generation Vaccines*

To address the issues outlined above, much effort has gone into developing safer smallpox vaccine candidates. Some studies aimed to generate vaccines using a sterile cell culture technique to reduce the risk of contamination by adventitious agents (second-generation vaccines) (see Table 1) [11]. For example, ACAM1000 [12,13] was propagated in MRC-5 cells (diploid human lung fibroblasts) using a single clone VV isolated from a Dryvax calf lymph vaccine (manufactured by Wyeth Laboratories using New York City Board of Health (NYCBH)). ACAM2000 was prepared in Vero cells under serum-free conditions using ACAM1000 as the seed virus [13,14]. The cell-cultured smallpox vaccine (CCSV), which was derived from a plaque-purified NYCBH strain, was also prepared in MRC-5 cells [15]. The Elstree-BN vaccine was manufactured in chicken embryo fibroblasts (CEF) using the Lister/Elstree

(Lister) strain, which was widely used as a lymph-derived vaccine in Europe, Africa and Asia during the global smallpox eradication campaign [16]. The manufacturing of vaccines in cell culture reduced the risk of vaccine contamination by extraneous agents. However, because second-generation vaccines were manufactured using first-generation vaccines or their isolates as seed viruses, their safety profiles were equivalent to those of the original lymph-derived vaccines, *i.e.*, they caused the same adverse events [13,15].

### *1.3. Third-Generation Vaccines*

As the global smallpox eradication campaign progressed and the risk of contracting smallpox infections diminished, developed countries began to raise concerns about the side effects associated with lymph-derived smallpox vaccines. This triggered new research to develop alternative attenuated vaccines (third-generation vaccines), such as the Modified Vaccinia Ankara (MVA) [17,18], Dairen I minute-pock variants (DIs) [19] and Lister Clone 16m8 (LC16m8) [20–23]. The main method used to attenuate the VVs was serial passage in primary cell culture or eggs.

MVA was attenuated by serial passage of the chorioallantois VV Ankara strain in CEF (>570 times). This resulted in the loss of approximately 15% of its genome, including host range-related genes, such as *K1L*, and some immunomodulatory genes, thereby generating a phenotype that is unable to replicate in most mammalian cells [24–26]. MVA, which shows an extremely attenuated phenotype in animals and humans [27], was administered to about 120,000 individuals in Western Germany and Turkey during the global eradication campaign with no apparent side effects [28]. Although its ability to protect against smallpox infection was not proven at that time, the need for a smallpox vaccine that was safe for use in immunocompromised individuals (including AIDS patients, patients treated with chemotherapy and transplant recipients) meant that MVA was examined in a number of clinical trials [29–33]. Data from these clinical trials and some animal experiments suggest that although MVA-derived vaccines have an extremely good safety profile; they are less immunogenic than replication-competent VVs, such as Dryvax, LC16m8 and LC16m8Δ [30,34–36]. For example, data from animal models show that multiple and 1–2 log higher doses of MVA are required to elicit antibody titers comparable with those elicited by replication-competent VVs [34–36].

The DIs strain originates from the Dairen I strain, a smallpox vaccine strain in Japan, and was isolated as a small-sized pock forming variant on chick chorioallantoic membrane (CAM) after 13 passages in one-day-old eggs [19]. As is the case with MVA, DIs harbors a large deletion within the left terminal region of the genome, which contains the host range genes, *K1L* and *C7L*, and the immunomodulatory gene, *K3L* [37]; consequently, DIs lacks the ability to replicate in a number of mammalian cell types. Although DIs showed a good safety profile when tested in field trials involving 200 Japanese children, it was not adopted as a smallpox vaccine, because it was less immunogenic than Lister Clone 16 (LC16).

Concerns regarding the side effects of first-generation smallpox vaccines, such as Ikeda, Dairen I and Lister were becoming a problem in Japan during the 1970s. In response to demands for a safer (but still effective) vaccine, the Chiba Serum Institute developed a highly attenuated strain, called LC16m8 [20,23]. LC16m8, which forms minute pocks on the CAM of embryonated eggs, was isolated from the Lister (Lister original, LO) strain via intermediate strains, such as LC16 and its derivative, LC16mO [23,38]. Tests in rabbit and monkey models showed that LC16m8 was markedly less neurovirulent than

first-generation vaccine strains, such as LO and Dryvax; indeed, its virulence was comparable with that of replication-defective DIs [21–23,39]. Moreover, LC16m8 induced a much weaker dermal reaction in rabbits and humans and showed a lower rate of febrile reactions than LC16mO (a direct parent of LC16m8) in clinical trials [23,40]. LC16m8 was administrated to approximately 100,000 infants without any serious adverse reactions and proved to be as immunogenic as the parental LO strain [23,40]. Therefore, LC16m8 was adopted as the favored vaccine strain in Japan [40].

### *1.4. Fourth-Generation Vaccines*

A number of novel attenuation approaches involving direct modification of the VV genome using genetic engineering techniques were used to develop highly attenuated VV strains (fourth-generation vaccines), such as NYVAC and LC16m8Δ [6,34,41–46]. These methods replaced classical attenuation methods based on serial passage in primary cell cultures or eggs. NYVAC was derived from the Copenhagen VV vaccine strain by deleting 18 non-essential genes, which include *C7L* and *K1L*, the host range genes; the thymidine kinase gene, a gene related to viral DNA synthesis and the *I4L* gene encoding the large subunit of ribonucleotide reductase. Thus, NYVAC shows very restricted replication in mammalian cells and a highly attenuated phenotype in animals [41]. However, since the replication of NYVAC in non-permissive mammal cells is arrested at an early stage [47] (as is the case for avipoxviruses, such as canary poxvirus and fowl poxvirus), it elicits weaker immune responses than MVA or replication-competent VVs [48].

LC16m8Δ should be categorized as a fourth-generation vaccine, because it was obtained from the parental smallpox vaccine strain (LC16m8) by deleting the *B5R* gene, which is responsible for the reversion of LC16m8. Consequently, it shows good genetic stability with very little (if any) reversion; however, it retains its ability to replicate in mammalian cells [34].

## 2. LC16m8 and *B5R*

Takahashi-Nishimaki *et al.* first identified the VV *B5R* gene, which is responsible for large-plaque formation and replication in Vero cells, during the course of investigating the mechanism of attenuation to generate LC16m8 [49]. LC16m8 harbors a frameshift mutation due to a single base deletion in the middle of its open reading frame (ORF); this mutation results in the loss of *B5R* function. *B5R* encodes a 42-kDa glycoprotein (B5 protein), which is involved in packaging the intracellular mature virion (IMV) within the trans-Golgi membrane or endosomal cisternae to form an intracellular enveloped virion (IEV) [50–52]. The IEV is transported along microtubules to the cell periphery [53,54], where it adheres to the cell membrane as a cell-associated enveloped virion (CEV). The B5 protein, in cooperation with the A36 and A33 proteins, also participates in the Src kinase-dependent formation of actin-containing microvilli and the subsequent release of the CEV from the cell surface to form an extracellular enveloped virion (EEV) [55,56]. Despite the relative paucity of whole progeny virions, EEVs play an important role in dissemination within the host [57]. Since anti-B5 antibodies neutralize EEV, *B5R*-expressing VV has been proposed as an effective smallpox vaccine [50,58–61].

When generating and performing basic research on LC16m8, we found that the vaccine spontaneously reverted to large-plaque-forming clones (LPCs) [34]. Thus, we were concerned that LPC contamination might ruin the safety profile of a future LC16m8 vaccine. Therefore, we investigated the

molecular mechanism(s) underlying the reversion, with a focus on the *B5R* gene associated with the formation of large plaques [49].

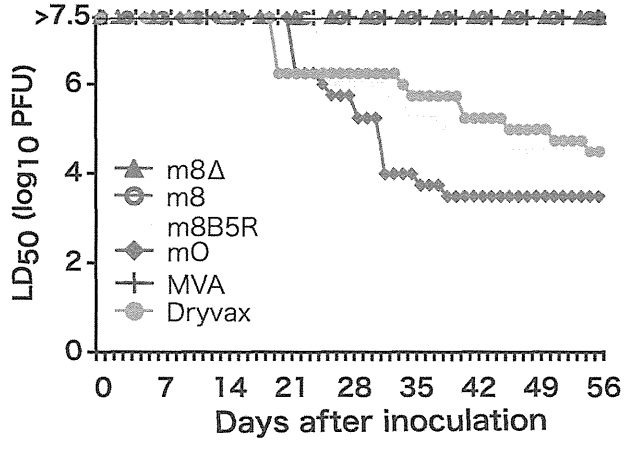
We isolated three LPC clones from a vaccine stock of LC16m8 and examined their phenotypes in terms of plaque size, dermal reactions in rabbits and pathogenicity in severe combined immunodeficiency (SCID) mice; these phenotypes were compared with those of LC16m8 and the parental virus LC16mO, which retains a fully-functional *B5R* gene. All three LPC viruses showed phenotypes similar to that of LC16mO, resulting in better growth in cell culture and greater virulence in SCID mice than LC16m8 [34]. As expected, sequencing the *B5R* in these LPCs revealed that the *B5R* ORF contained a single base insertion upstream of the nucleotide that was deleted from the LC16m8 *B5R*. It is noteworthy that the nucleotide insertion site in the LPC *B5R* ORF was different in each of the three clones, even though they originated from the same viral stock, which was prepared through only seven passages after the LC16m8 cloning. These results strongly suggest that the reversion of LC16m8 is a multi-clonal event and may occur frequently.

## 3. LC16m8Δ

### *3.1. Safety Profile*

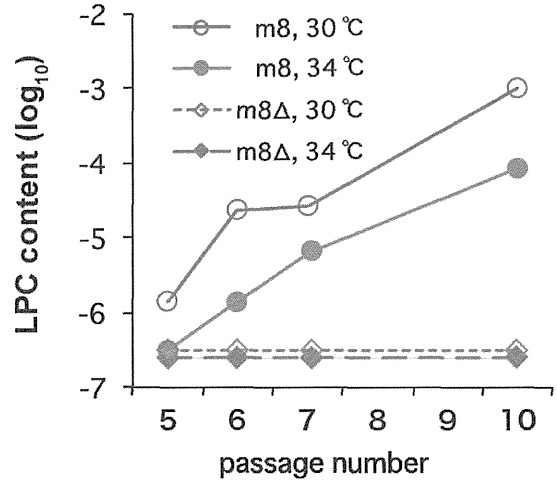
To prevent the generation of LC16m8 revertants, we decided to delete the entire *B5R* gene from the LC16m8 genome by homologous recombination to yield LC16m8Δ [34]. The phenotype of LC16m8Δ (plaque size and dermal reaction in rabbits) was similar to that of LC16m8. Intraperitoneal (i.p.) injection of $10^7$ PFU of LC16m8Δ (a dose three logs higher than that needed to elicit protective immunity in BALB/c mice) did not cause any symptoms in SCID mice over an eight-week period (Figure 1). MVA and plaque-purified LC16m8 (which contains a very low level of revertants) were also nonpathogenic; however, LC16mO and m8*B5R* (a derivative constructed by reintroducing the intact *B5R* gene into LC16m8) caused severe rashes and death in SCID mice, even when administered at a dose two logs lower than LC16m8Δ (Figure 1).

**Figure 1.** Pathogenicity of *B5R*-defective viruses in severe combined immunodeficiency (SCID) mice. Figure modified from Kidokoro *et al.* [34].



The genetic stability of LC16m8Δ was evaluated by serial passage in primary rabbit kidney (PRK) cells, which were used to generate the LC16m8 vaccine. No detectable LPCs emerged from LC16m8Δ under any of the test conditions, including those used in vaccine production (passage in PRK cells at 30 °C). By contrast, LPCs emerged from LC16m8 that was plaque-purified immediately before testing (Figure 2). It should be noted that once LPCs appeared in the cultures, the LPCs:LC16m8 ratio increased rapidly with the passage number (Figure 2).

**Figure 2.** Genetic stability of LC16m8Δ and LC16m8 upon serial passage in primary rabbit kidney cells at different temperatures (30 °C or 34 °C). Figure modified from Kidokoro *et al.* [34]. LPC, large-plaque-forming clone.

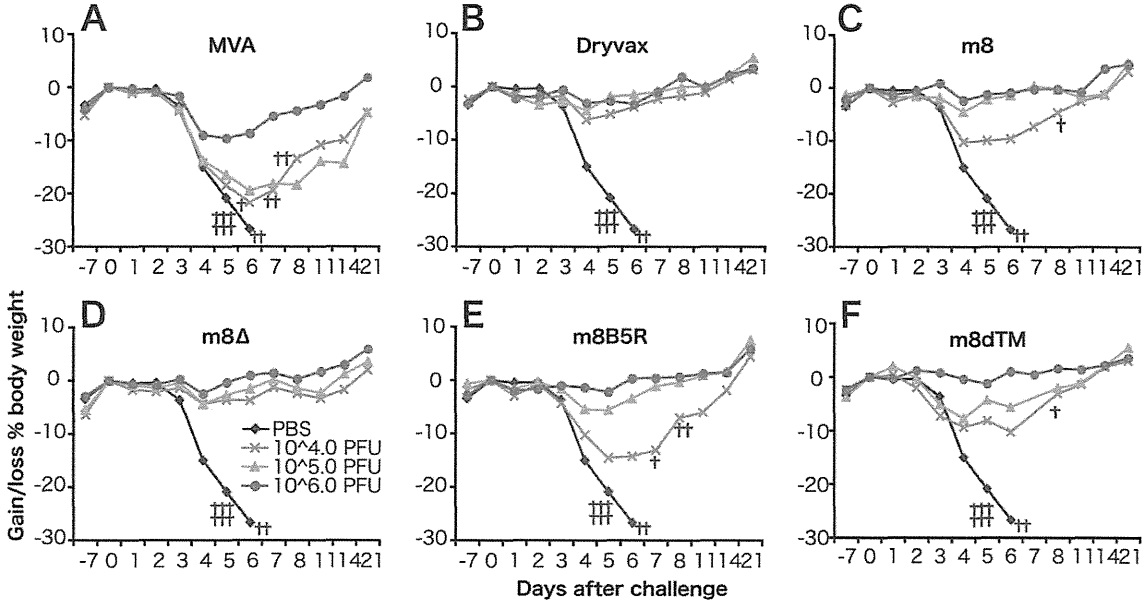



*3.2. Immunogenicity*

The protective immune response elicited by LC16m8Δ was compared with that elicited by Dryvax, MVA, LC16m8 and LC16m8 derivatives (m8*B5R* and m8dTM, both of which express the B5 ectodomain at high levels) in a mouse model. This model, in which the immunized mice are challenged with a highly pathogenic VV (the Western Reserve (WR) strain), is one of the most popular methods of evaluating the efficacy of smallpox vaccines [62] (Figure 3). We immunized each group of mice with a single dose ($10^4$, $10^5$ or $10^6$ PFU) of each VV via the intramuscular (i.m.) route. We found that the level of protective immunity elicited by LCm8Δ was comparable with that elicited by Dryvax and superior to that elicited by MVA. For example, the minimal dose ($10^4$ PFU) of LC16m8Δ or Dryvax fully protected mice from lethal infection with WR, whereas mice immunized with MVA, LC16m8, m8*B5R* or m8dTM, lost weight and, in some cases, died. The maximum dose ($10^6$ PFU) of MVA resulted in prominent weight loss after WR challenge. It is noteworthy that immunization with LCm8Δ was more efficient than that with m8*B5R* or m8dTM when compared at their minimal dose. In particular, m8*B5R* was significantly inferior to LC16m8Δ (*t*-test, $p = 0.005$). These results suggest that *B5R* does not play a major role in eliciting protective immune responses in these mice. In addition, LC16m8Δ elicited protective immune responses in cynomolgus monkeys and fully protected them against lethal infection with monkeypox virus [63]. Taken together, these data suggest that LC16m8Δ is as effective as the first-generation smallpox vaccine, Dryvax. Although several studies report that the B5 protein is the major target of EEV-neutralizing antibodies, which are significant for protection against smallpox

infection, immunization with B5-deficient vaccine viruses protects animals against lethal challenge by pathogenic orthopoxviruses [58,64–67]. In addition, some reports show that smallpox vaccines do not always induce anti-B5 antibodies, and antibody response profiles against each viral protein are highly heterologous in humans [68–70]. They also concluded that the key to inducing a strong neutralizing antibody response is to elicit antibodies that recognize multiple viral proteins; these antibodies then act synergistically to provide better protection.

**Figure 3.** (A) Protective immune responses induced by m8Δ and derivative viruses in mice. (A–F) Average body weight of mice immunized (intramuscularly) with ($10^4$–$10^6$ PFU) vaccinia viruses (VVs) and then challenged intranasally with the Western Reserve (WR) strain. Crosses denote mice that either died or were sacrificed because they lost >30% of their body weight. Figure modified from Kidokoro *et al.* [34].




## 4. LC16m8Δ as a Vehicle for Expressing Foreign Genes

VV has been widely used as a vector for expressing foreign genes, because it has many excellent properties: high expression efficiency, a broad host range, a very large capacity for accepting foreign genes, heat stability and inexpensive vaccine production [71]. Therefore, VV vectors have been examined for use as live vaccines against both human and veterinary infectious diseases and cancers [6–8]. However, concerns about the safety profile of VVs are a major barrier to developing recombinant VV vaccines for use in humans [72].

Most research has focused on replication-defective poxvirus vectors (which have better safety profiles) as vehicles for delivering antigens derived from human pathogens. For example avipox- [73], MVA- and NYVAC-based vectors expressing components of human pathogens, such as HIV-1 and tuberculosis, have been developed and evaluated in monkeys [74,75] and humans [76–79]. However, although promising in animal models, these vaccines did not induce sufficiently strong immune responses in humans, nor did they protect humans from infection [79,80]. Therefore, more effective vehicles are needed for human vaccine development.

Thus, a replication-competent VV that has been proven to be safe for human vaccination against smallpox could be a good candidate. The safety profile and strong antigenicity of LC16m8Δ, a genetically-stable variant of LC16m8, make it a promising vehicle for a vaccine against HIV or other human diseases.

One concern regarding the use of viral vectors is pre-existing immunity against the vector virus, which has the potential to dampen specific immune responses. However, Kohara *et al.* showed that a recombinant LC16m8 vaccine expressing the SARS coronavirus (SARS-CoV) spike protein elicited neutralizing antibodies against SARS-CoV in rabbits that generated a high titer of anti-LC16m8 antibodies [81]. Another report shows that the VV lacking the B5 ectodomain induces a more potent immune response in vaccinia-immune animals than its wild-type counterpart [82]. These results suggest that LC16m8Δ would make a good vector virus for eliciting effective immune responses against foreign antigens in individuals pre-immunized with smallpox vaccines.

Previously, we developed the pSFJ1-10 promoter, an A-type inclusion body (ATI) complex promoter that comprises ten repeat units of the mutated early region of the p7.5 promoter plus the ATI late promoter [83]. This complex promoter possesses strong activity in both the early and late phases of the VV infection cycle. Indeed, the H protein of the measles virus and chloramphenicol acetyltransferase, the synthesis of which is driven by this promoter, comprised approximately 10% of total cellular protein [84,85]. Moreover, we constructed LC16m8ΔVNC110, a vector that harbors pSFJ1-10 along with a multiple cloning site within the hemagglutinin (HA) gene, which can be used for the rapid production of recombinant LC16m8Δ through *in vitro* ligation of the LC16m8ΔVNC110 genome with foreign DNA [86]. The foreign genes inserted were stably maintained in the LC16m8Δ recombinants constructed by this technique and harboring the p7.5 promoter after several passages in the RK13 cells, a standard cell line for the propagation of VVs.

Using this technique, we tested whether LC16m8Δ is a better vector than non-replicating vaccinia virus for the expression of SIV Gag. The Gag proteins of HIV-1 and SIV are major antigens that elicit cytotoxic T lymphocyte (CTL) responses. The activity of anti-Gag CTL inversely correlates with the viral load in HIV-1-infected individuals [87]. Experimental infection of monkeys with SIV suggests that the strength of the anti-Gag CTL response correlates with the containment of SIV [88]. A m8Δ/pSFJ/SIVGag vector expressing the SIV Gag antigen under control of the pSFJ1-10 promoter generated significantly more Gag protein *in vitro* and elicited the production of anti-Gag IFN-$\gamma^+$ T-cells in mice, more efficiently than the non-replicating VV DIs strain (which harbors the *gag* gene under the control of the same promoter) [86]. The DIs strain is immunogenically similar to MVA [89].

We further optimized LC16m8Δ for use as a vector by comparing the immunogenicity of SIV Gag proteins expressed under the control of either the pSFJ1-10 promoter or the p7.5 promoter, which is a classical early-late promoter [90] with moderate activity (although weaker than that of pSFJ1-10). Preliminarily observations indicated that expressing too much foreign protein led to a reduction in VV propagation *in vitro*; therefore, the balance between the expression of a foreign antigen and viral propagation *in vivo* might be crucial for optimal immunogenicity. Thus, we compared the immunogenicity and virulence of m8Δ/p7.5/SIVGag with that of m8Δ/pSFJ/SIVGag in the setting of a recombinant Bacillus Calmette-Guerin (BCG) prime/recombinant LC16m8Δ boost vaccination protocol. This setting was based on the observation that long-term maintenance of effector memory T-cells (Tem) with the capacity to immediately attack SIV-infected cells restricts infection by antibody-resistant SIV at the site

of virus entry. This was achieved using vaccine approaches that persistently express viral antigens in vaccinated macaques via the use of a cytomegalovirus (CMV) vector, thereby resulting in continuous immune stimulation [91,92]. Since BCG persists in vaccinated individuals for long periods of time (up to 10 years) without serious symptoms, vaccination with BCG expressing the Gag protein may be expected to induce Gag-specific $CD8^+$ T-cells and to maintain immunological memory (via Tem) for a long time. Vaccination studies in mice revealed that m8Δ/pSFJ/SIVGag was less pathogenic and elicited Gag-specific IFN-$\gamma^+$, CD107$\alpha^+$ and $CD8^+$ T-cells more efficiently than m8Δ/p7.5/SIVGag. Tem were detected even at four months after boosting with m8Δ/pSFJ/SIVGag. Therefore, LC16m8Δ that express SIV Gag under the control of the pSFJ1-10 promoter induced more efficient and long-lasting immune responses than LC16m8Δ harboring the p7.5 promoter [93].

Although inducing both anti-HIV-1 antibody and cytotoxic $CD8^+$ T-cells is an effective way of preventing HIV-1 infection, it is often difficult to induce the production of anti-HIV-1 antibodies, particularly neutralizing antibodies, at a high titer. For example, only a low titer of anti-HIV-1 Env antibodies was observed, even after repeated immunization with an MVA-based vector [94]. Repetitive antigenic stimulation is required for affinity maturation, the process by which high avidity neutralizing antibodies against HIV-1 are generated. Long-lasting expression of antigen by a replication-competent vector, such as LC16m8Δ, may enable repeated immunological presentation, which induces affinity maturation.

We next examined the ability of LC16m8Δ expressing the HIV-1 Env gene to elicit anti-HIV-1 antibodies and $CD8^+$ T-cells in mice in the setting of a recombinant LC16m8Δ prime followed by a Sendai virus vector boost. We found that this vaccination regimen led to the efficient induction of both Env-specific $CD8^+$ T-cells and anti-Env antibodies, including neutralizing antibodies. These results are in sharp contrast to those reported by studies that used vaccine regimens based on priming with an Env-expressing plasmid followed by a boost with the LC16m8Δ or SeV vector; such an approach mainly induced cell-mediated immune responses [95].

## 5. Conclusions

Despite its replication-competent phenotype, LC16m8Δ is highly attenuated and shows no pathogenic effects in SCID mice (similar to replication-defective VVs, such as MVA). However, it is a comparably effective smallpox vaccine with respect to Dryvax. Moreover, LC16m8Δ-based vectors induce both antibody- and cell-mediated immune responses against foreign antigens more efficiently than non-replicating VV vectors. Therefore, LC16m8Δ is superior to non-replicating VV vectors and is suitable for use in humans. We also point out that LC16m8Δ recombinants may be useful as a dual vaccine against both smallpox and pathogens targeted with the inserted genes.

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