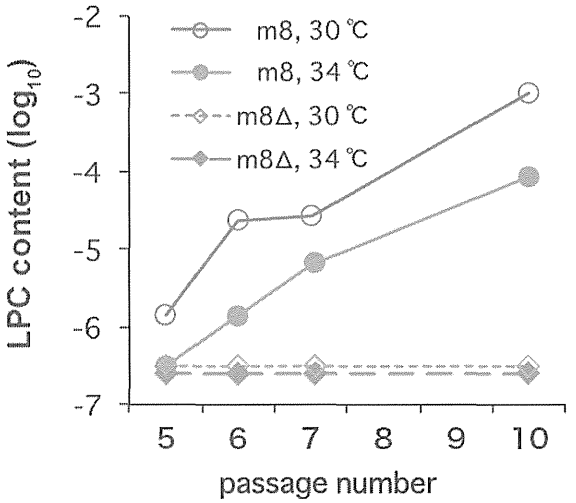
The genetic stability of LC16m8Δ was evaluated by serial passage in primary rabbit kidney (PRK) cells, which were used to generate the LC16m8 vaccine. No detectable LPCs emerged from LC16m8Δ under any of the test conditions, including those used in vaccine production (passage in PRK cells at 30 °C). 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.

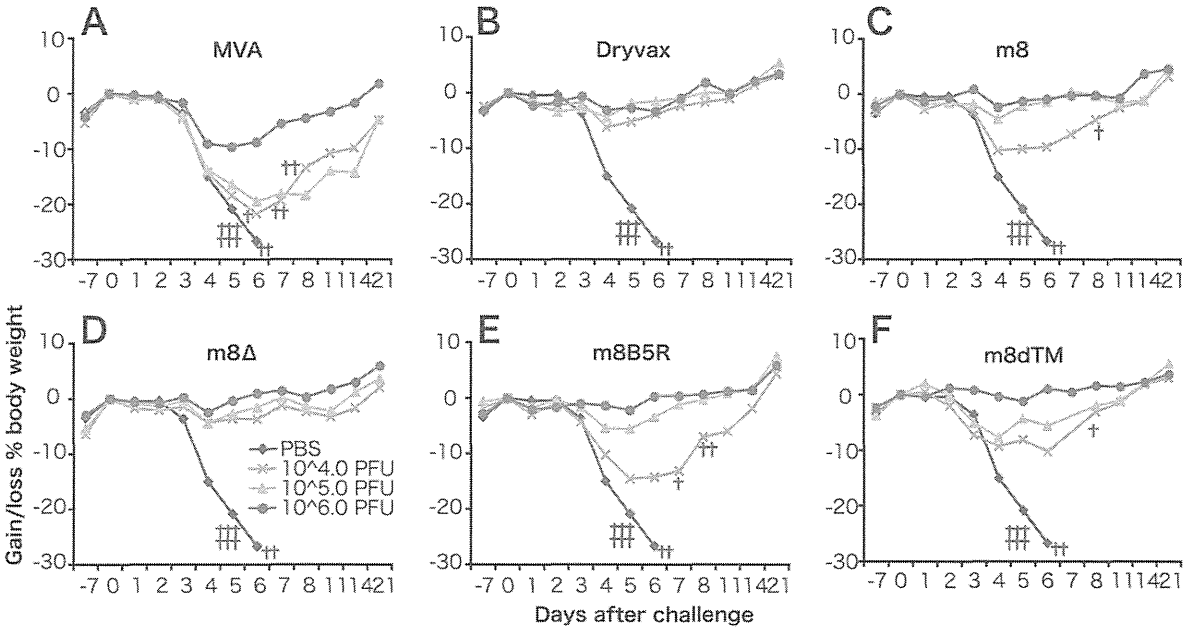


3.2. Immunogenicity

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-γ^+ 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-γ^+, CD107α^+ 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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Author Contributions

Both authors contributed equally to this work.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Combined Cytolytic Effects of a Vaccinia Virus Encoding a Single Chain Trimer of MHC-I with a Tax-Epitope and Tax-Specific CTLs on HTLV-I-Infected Cells in a Rat Model

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Adult T cell leukemia (ATL) is a malignant lymphoproliferative disease caused by human T cell leukemia virus type I (HTLV-I). To develop an effective therapy against the disease, we have examined the oncolytic ability of an attenuated vaccinia virus (VV), LC16m8Δ (m8Δ), and an HTLV-I Tax-specific cytotoxic T lymphocyte (CTL) line, 4O1/C8, against an HTLV-I-infected rat T cell line, FPM1. Our results demonstrated that m8Δ was able to replicate in and lyse tumorigenic FPM1 cells but was incompetent to injure 4O1/C8 cells, suggesting the preferential cytolytic activity toward tumor cells. To further enhance the cytolysis of HTLV-I-infected cells, we modified m8Δ and obtained m8Δ/RT1AlSCTax180L, which can express a single chain trimer (SCT) of rat major histocompatibility complex class I with a Tax-epitope. Combined treatment with m8Δ/RT1AlSCTax180L and 4O1/C8 increased the cytolysis of FPM1V.EFGFP/8R cells, a CTL-resistant subclone of FPM1, compared with that using 4O1/C8 and m8Δ presenting an unrelated peptide, suggesting that the activation of 4O1/C8 by m8Δ/RT1AlSCTax180L further enhanced the killing of the tumorigenic HTLV-I-infected cells. Our results indicate that combined therapy of oncolytic VVs with SCTs and HTLV-I-specific CTLs may be effective for eradication of HTLV-I-infected cells, which evade from CTL lysis and potentially develop ATL.

1. Introduction

Human T cell leukemia virus type I (HTLV-I) is etiologically linked to adult T cell leukemia (ATL) [1, 2] and a chronic progressive neurological disorder termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3, 4]. HTLV-I genome contains a unique 3′ region, designated as pX, which encodes the viral transactivator protein, Tax [5]. It is speculated that Tax plays a central role in HTLV-I associated immortalization and transformation of T cells, which may lead to the development of ATL [6]. In addition, Tax is also known as a major target protein recognized by cytotoxic T lymphocyte (CTL) of HTLV-I carriers [7]. A number of studies have reported that CTL responses were activated in HAM/TSP patients but were weak in ATL patients, suggesting that the T cell response could be one of the important determinants of the disease manifestation [8]. Since HTLV-I Tax-specific CTL can recognize and lyse ATL cells in vitro [9], it is conceivable that the low CTL activity in ATL patients is disadvantageous as it may allow uncontrolled proliferation and evolution of HTLV-I-infected cells in vivo. Indeed, Hasegawa et al. have reported that oral HTLV-I-infection induced HTLV-I-specific T cell tolerance and caused an elevation of the proviral loads and that reimmunization resulted in the recovery of the virus-specific T cell responses and the decrease of the proviral loads in a rat model system [10]. In addition, the development of ATL has been reported in HTLV-I carriers who received immunosuppressants during organ transplantation [11]. Increase of Tax-specific CTLs observed in ATL patients treated successfully

with allogeneic hematopoietic stem cell transplantation (allo-HSCT) also suggests the importance of virus-specific CTLs to control the disease [12]. Thus, immune therapies to activate HTLV-I-specific CTLs are considered as novel attempts for the treatment of ATL. In this regard, we have previously demonstrated the therapeutic effect of Tax-coding DNA or peptide in a rat model of ATL-like disease [13, 14]. In addition, it has been recently reported that autologous Tax-specific CTLs showed therapeutic benefits in an animal model using NOG mice bearing primary ATL cells, suggesting the possible translation into a clinical use [15].

To improve therapeutic effects of immune therapy, it is important to consider tumor microenvironment, because tumor cells often induce a microenvironment, which favors the development of immunosuppressive populations of immune cells, such as myeloid-derived suppressor cells and regulatory T cells [16]. In HTLV-I carriers and ATL patients, various kinds of immunosuppressive events have been reported, indicating the importance of developing new strategies to eliminate HTLV-I-infected cells in such immunosuppressive environments [8]. One of powerful strategies to lyse tumor cells in an immunosuppressive microenvironment would be the use of replication-competent oncolytic viruses, because oncolytic virotherapy has been known to induce both direct tumor killing and local proinflammatory environments that help to reverse the immunosuppressive environment of tumors [17, 18]. As for HTLV-I infection, vesicular stomatitis virus (VSV) has been reported to have oncolytic activity against primary ATL cells [19]. Vaccinia virus (VV) has been also shown to be a good candidate for oncolytic virotherapies [20]. It has been already assessed in clinical trials and shown to selectively infect, replicate, and express transgene products in cancer tissues without damaging normal tissues [21]. We have previously constructed a highly attenuated VV, LC16m8Δ (m8Δ), which is genetically more stable than LC16m8 (m8), a naturally occurring counterpart of m8Δ, and less pathogenic than its parental LC16mO (mO) due to the deletion of B5R gene [22]. The safety of m8Δ has been already confirmed in clinical use of its natural counterpart; m8 has been safely administered to approximately 100,000 infants and 3,000 adults for smallpox vaccination and induced levels of immunity similar to those of the original Lister strain without serious side effects [23]. Moreover, Hikichi et al. have recently reported the oncolytic potential of m8Δ with regulated expression of B5R [24]. Thus, the application of m8Δ for the elimination of HTLV-I-infected cells should be possible.

It is well known that HTLV-I viral protein expression is suppressed in infected cells in the peripheral blood of the virus-infected individuals, probably due to either unidentified suppression mechanisms of HTLV-I expression or genetic and epigenetic changes in the viral genome [8]. This reduction of viral protein expression may cause the decrease of anti-HTLV-I immune responses. Downregulation of major histocompatibility complex class I (MHC-I) could also lead to the evasion of HTLV-I-infected cells from the virus-specific CTLs [25]. Thus, strategies to overcome the repression of viral antigen presentation in HTLV-I infected individuals should be also required to establish effective anti-HTLV-I therapies. Improving the ability to present antigen to proper CTLs could be one possible way to overcome the problem. Single chain trimers (SCTs) of MHC-I have been reported to possess the strong potential to stimulate antigen-specific T cells [26, 27]. In this system, all three components of MHC-I complexes, such as an antigen peptide, β_2-microgrobulin (β_2m), and MHC-I heavy chain, were covalently attached with flexible linkers. By connecting together the three components into a single chain chimeric protein, a complicated cellular machinery of antigen processing can be bypassed, leading to stable cell surface expression of MHC-I coupled with an antigenic peptide of interest. It has been recently reported that SCT-expressing DNA vaccine is able to break immune tolerance against self-antigen from melanoma, further supporting the potential of SCTs to clinical applications [28]. By applying SCT system to a rat model of HTLV-I infection, we have previously developed an activation and detection system of Tax-specific rat T cells and showed that SCTs with a Tax-epitope specifically recognize and activate Tax-specific CTLs [29]. In this study, to further improve the efficacy of CTL activation by SCTs, m8Δ was selected as a vector to express SCTs on the surface of HTLV-I-infected cells. Introduction of SCT coding sequence into the genome of m8Δ could generate novel therapeutic VVs, which possess abilities to both lyse tumor cells and activate tumor-specific CTLs. We further examined the combination effects of Tax-specific CTLs and m8Δ expressing SCT against CTL-resistant HTLV-I-infected cells. Our results suggested the possible application of the combined use of oncolytic viruses presenting tumor antigens and tumor-specific CTLs for the treatments against tumors including ATL.

2. Materials and Methods

2.1. Cells and Viruses. An HTLV-I-immortalized cell line, FPM1, was previously established by cocultivating thymocytes of an F344/N Jcl-rnu/+ rat (Clea Japan, Inc. Tokyo, Japan) with HTLV-I producing human cell line, MT-2, which was treated with mitomycin C (50 μg/mL) for 30 min at 37°C [30]. FPM1V.EFGFP, FPM1V.EFGFP/8R, and 4O1/C8 cells were established as previously described [25]. FPM1V.EFGFP was a subclone of FPM1 cells, which stably expresses EGFP. FPM1V.EFGFP/8R cells were generated from FPM1V.EFGFP cells, by continuously cultivating with a Tax-specific CTL, 4O1/C8, and obtained an ability to evade from CTL killing by 4O1/C8 cells. FPM1 and its subclones were maintained in RPMI 1640 with 10% heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO), 55 μM of 2-mercaptoethanol (Gibco, Grand Island, NY), penicillin, and streptomycin. The 4O1/C8 cells were established from an F344/N Jcl-rnu/+ rat inoculated with Tax-coding DNA and were maintained in RPMI 1640 medium with 10% FCS, 55 μM of 2-mercaptoethanol, and 20 U of IL-2 (PEPROTECH, London, UK) per mL with periodical stimulation using formalin-fixed FPM1 cells every 4 weeks. A rabbit kidney epithelial cell line, RK13, was cultured in RPMI1640 supplemented with 10% FCS. Hamster BHK cells were cultured in D-MEM supplemented with 10% FCS. Canarypox virus (a kind gift of National Institute of Animal Health) [31], mO, m8Δ [22], and LC16 m8ΔVNC110 that harbors multiple cloning site in the HA gene of m8Δ

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-β_2m-RT1A^l 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 μg/mL aprotinin, and 1 μg/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 μg 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

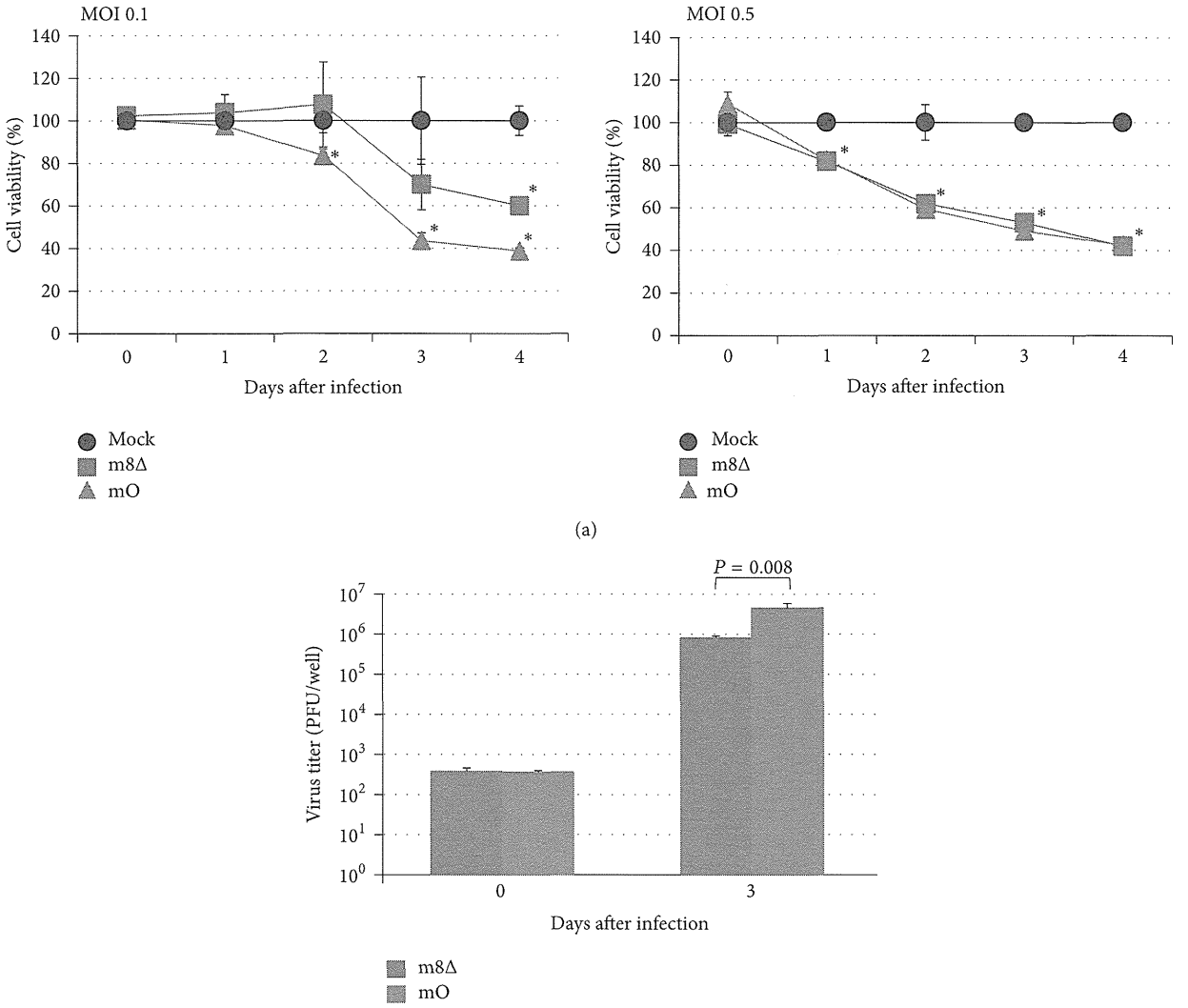

FIGURE 1: Viability of FPM1 cells infected with attenuated VVs. (a) FPM1 cells were exposed to m8Δ (■), mO (▲), or PBS (●) at indicated MOI for 2 hrs. After extensive wash, the cells were cultured for indicated periods and the cell growth was assessed by using cell counting kit 8. The cell viabilities 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

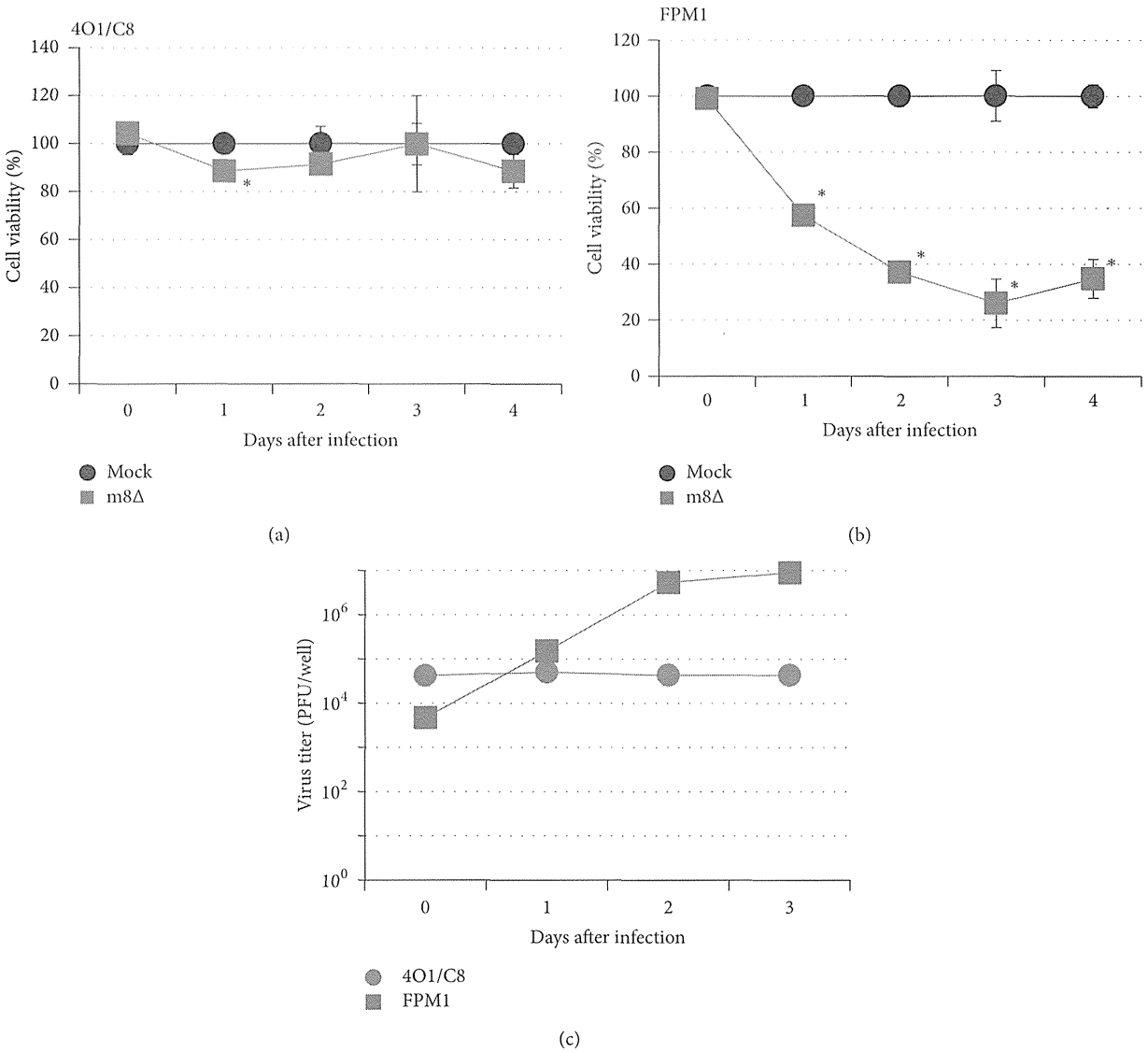

FIGURE 2: A Tax-specific CTL line, 4O1/C8, was resistant to killing by m8Δ. The 4O1/C8 (a) or FPM1 (b) cells were exposed to m8Δ (■) or PBS (●) at MOI 2 for 2 hrs. After extensive wash, the cells were cultured for indicated periods and the cell growth was assessed by using cell counting kit 8. The cell 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-γ production in the supernatants was evaluated. As shown in Figure 3(b), mixed culture of 4O1/C8 cells with FPM1V.EFGFP induced IFN-γ secretion whereas that with FPM1V.EFGFP/8R did not. Thus, production of IFN-γ 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

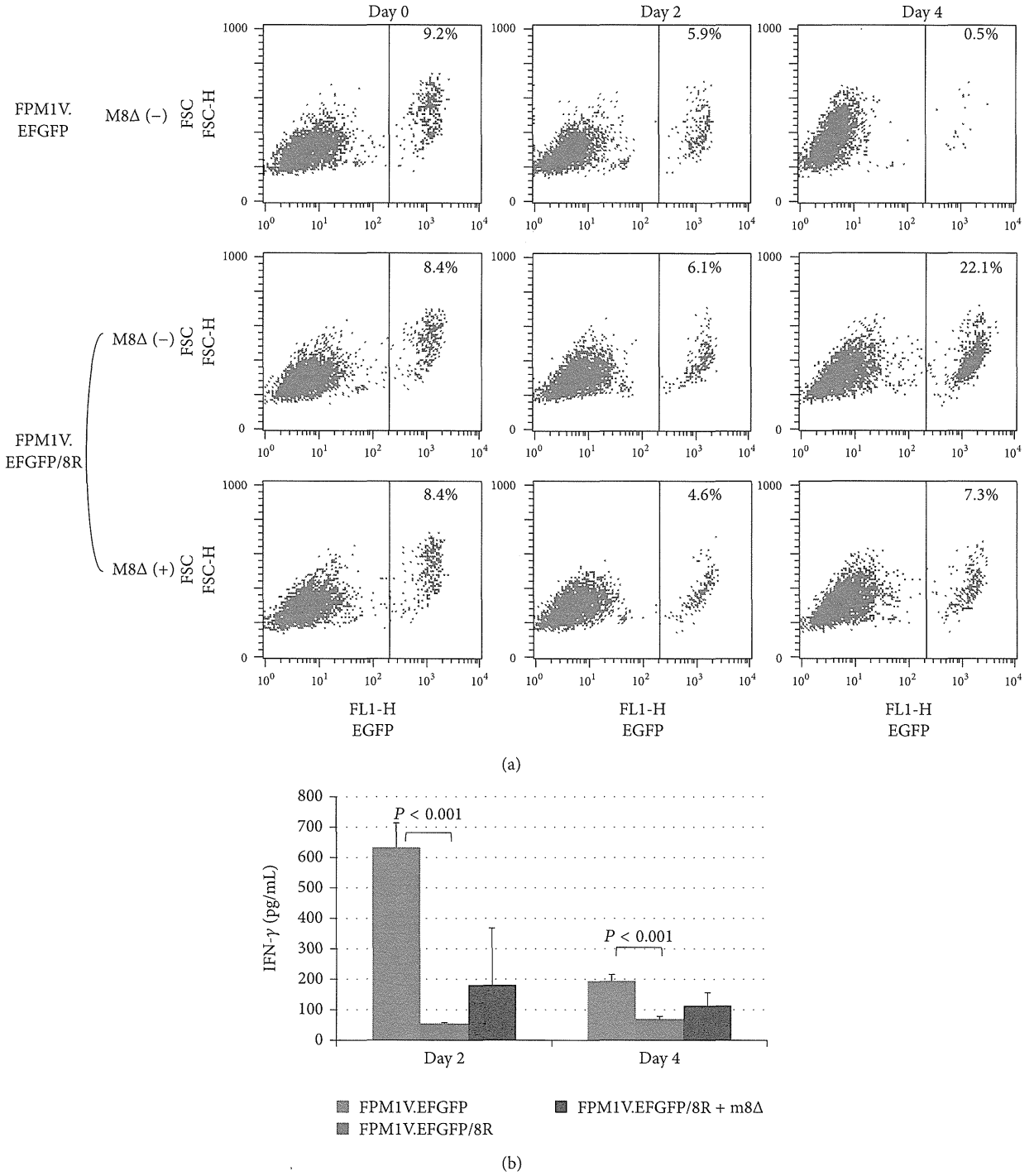

FIGURE 3: Lack of IFN-γ production was correlated with the resistance of FPM1V.EFGFP/8R cells to killing by 4O1/C8. (a) FPM1V.EFGFP or FPM1V.EFGFP/8R cells (5×10^4/well) were mixed with 4O1/C8 cells (5×10^5/well) at an E : T ratio of 10 : 1 in the presence or absence of m8Δ (1×10^5 PFU/well) and subjected 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-γ 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-γ 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-γ secretion by 4O1/C8. In contrast, RK13 cells infected with m8Δ/RT1AlSCNLEnv371L induced little amount of IFN-γ 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-γ 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-γ production was clearly induced in the mixed culture (Figure 6). Induction of IFN-γ 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-γ (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

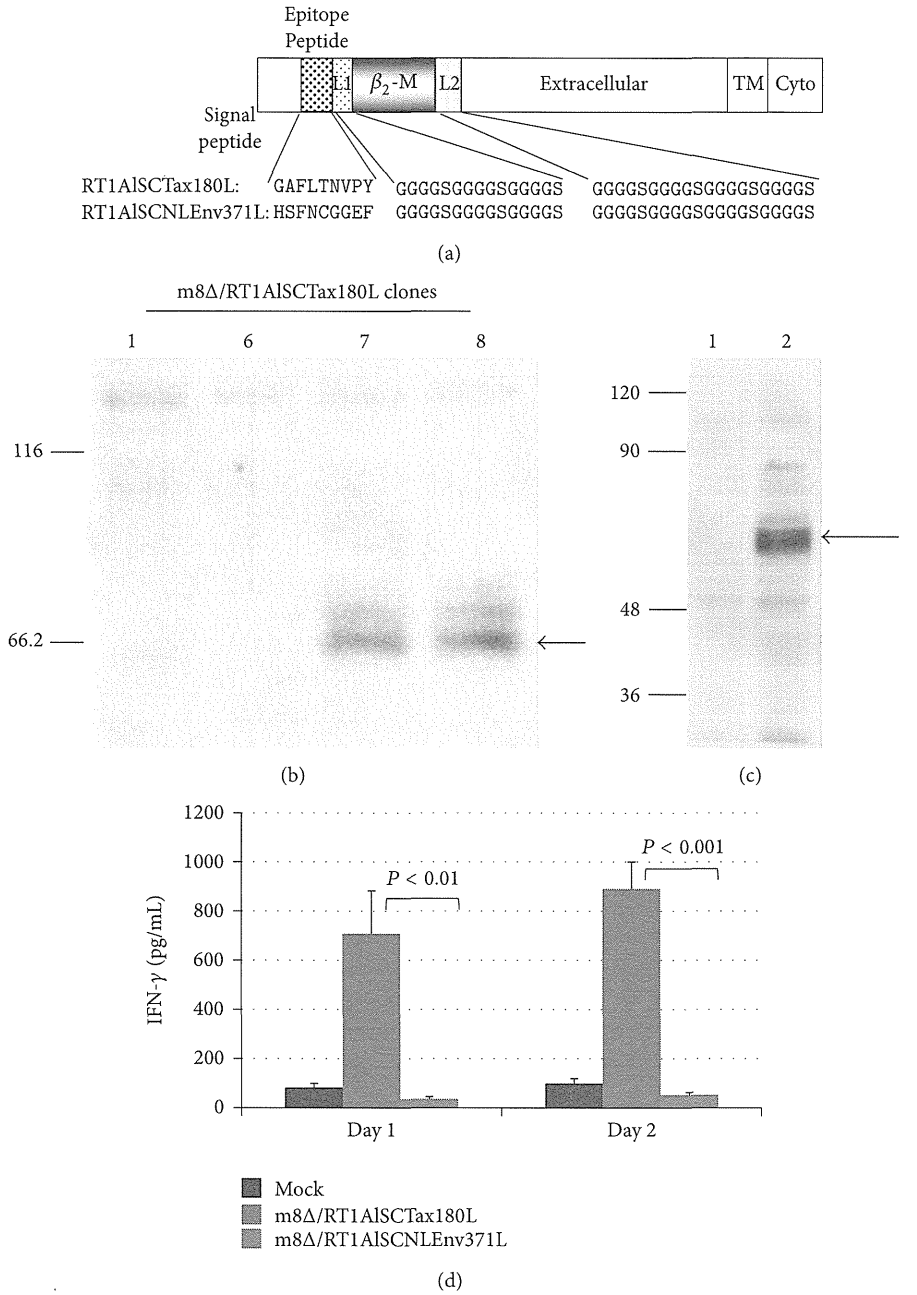

FIGURE 4: Characterization of m8Δ expressing SCTs. (a) Diagram of SCTs encoding Tax180–188 or NLEnv371–379 linked to β_2m and RT1.A^l molecules with two linkers. L1, linker 1; L2, linker 2; TM, transmembrane region; Cyto, cytoplasmic region. ((b) and (c)) Whole cell extracts were isolated from RK13 cells infected with indicated clones of m8Δ/RT1AlSCTax180L (b) or RK13 cells infected with m8Δ (lane 1) or m8Δ/RT1AlSCNLEnv371L (lane 2) (c) and 50 μg 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-γ 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,