Selective killing of Kaposi's sarcoma-associated herpesvirus lytically infected cells with a recombinant immunotoxin targeting the viral gpK8.1A envelope glycoprotein

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Kaposi sarcoma-associated herpesvirus (KSHV, human herpesvirus 8) is etiologically associated with three neoplastic syndromes: Kaposi sarcoma and the uncommon HIV-associated B-cell lymphoproliferative disorders primary effusion lymphoma and multicentric Castleman disease. The incidence of the latter B-cell pathology has been increasing in spite of antiretroviral therapy; its association with lytic virus replication has prompted interest in therapeutic strategies aimed at this phase of the virus life cycle. We designed and expressed a recombinant immunotoxin (2014-PE38) targeting the gpK8.1A viral glycoprotein expressed on the surface of the virion and infected cells. We show that this immunotoxin selectively kills KSHV-infected cells in dose-dependent fashion, resulting in major reductions of infectious virus release. The immunotoxin and ganciclovir, an inhibitor of viral DNA replication, showed marked reciprocal potentiation of antiviral activities. These results suggest that the immunotoxin, alone or in combination, may represent a new approach to treat diseases associated with KSHV lytic replication.

Introduction

Kaposi sarcoma-associated herpes virus (KSHV, human herpes virus 8) is a human oncogenic DNA virus belonging to the gammaherpesviridae family, rhadinoviridae genus.[1] Like all herpesviruses[2] KSHV displays two distinct transcriptional programs, latency and lytic replication. The KSHV latency phase is associated with restricted gene expression involved in maintenance of the viral genome as a nuclear episome, cell proliferation, immune evasion and suppression of apoptosis; the lytic phase involves temporal expression of genes leading to virus replication and infectious virion release.[3]

Since its initial identification in 1994 by representational differential analysis of AIDS-related Kaposi sarcoma (KS) tissues,[4] KSHV was subsequently implicated in two uncommon, typically human immunodeficiency virus (HIV)-associated, B-cell lymphoproliferative disorders—primary effusion lymphoma (PEL),[5] and the plasmablastic variant of multicentric Castleman disease (MCD).[6] Its etiological role in all three diseases is now firmly established.[7-10] Among these KSHV-associated pathologies, there are marked differences in the patterns of latent vs. lytic phase gene expression as judged by protein expression patterns,[11,12] quantitative DNA analysis coupled with computerized immunohistochemistry,[13] and serum antibody profiles.[14] MCD is distinctive for the prominent involvement of KSHV lytic phase replication. The plasmablastic form involves monotypic (IgM, λ) polyclonal B-cell proliferation accompanied by episodic life-threatening flare-ups of fever, peripheral lymphadenopathy, splenomegaly, severe cytopenia and elevated levels of interleukin-6 (including the virus-encoded homolog) and other inflammatory markers.[15-18] Consistent with the involvement of lytic phase replication, high viral loads in peripheral blood mononuclear cells accompany acute episodes,[19] and high level KSHV DNA in plasma has been proposed as a predictor of an active attack.[20] Oddly while the incidence of HIV-associated KS has declined with access to highly active antiretroviral therapy (HAART),[21] HIV-associated MCD incidence has been increasing despite HAART.[22]
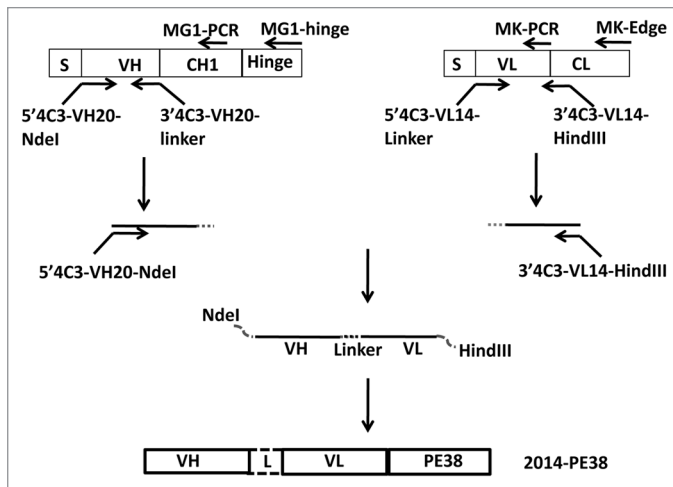
Figure 1. Schematic of construction of the 2014-PE38 immunotoxin expression plasmid and relative positions of the PCR primers used for the cloning procedure. The V_H and V_L segments of anti-KSHV K8.1A mAb 4C3 were linked by a 15 amino acid linker $(G_4S)_3$ and ligated in frame to PE38.

Plasmablastic MCD is a highly aggressive syndrome; left untreated, it is generally fatal within two years, with median survival around 1 y.[23] While standardized treatment modalities have not yet been established, several promising approaches are being pursued.[16,24,25] Antineoplastic chemotherapy with single or combination agents has yielded clinical remissions, though the responses are typically transient and drug toxicities limit extended use. Successes have been reported with immunotherapy using monoclonal antibodies (mAbs) tocilizumab (Actemra®),[26] and siltuximab[27] against interlekin-6 or rituximab against the B-cell antigen CD20,[18,28-31] though the latter agent has been complicated by aggravation of KS lesions.[28,29,32] A recent report described MCD remission in a multiple myeloma patient undergoing treatment with the proteosome inhibitor bortezomib.[33] Finally, herpesvirus-directed treatments using inhibitors of the viral DNA polymerase have shown promise,[34,35] consistent with the involvement of lytic phase replication in MCD; however, as for chemotherapy, the benefits are transient and long-term use is complicated by dose-limiting toxicities of these agents.

As a potential therapeutic intervention against MCD, we propose specific killing of KSHV-infected cells using targeted cytotoxic proteins (toxic moieties linked to mAbs or ligands) that specifically recognize a KSHV-encoded glycoprotein displayed on the infected cell surface. This concept, which has been vigorously pursued for decades in the oncology field, exploits three variations of the theme of targeted cytotoxic proteins:[36] (1) antibody-drug conjugates (ADCs) in which mAbs are chemically coupled to low molecular weight cytotoxic compounds,[37,38] (2) radioimmunotherapy whereby radionuclide-conjugated mAbs deliver lethal doses of radiation to the targeted cells,[39,40] and (3) immunotoxins wherein the antigen-binding regions of mAbs are linked (chemically or genetically) to the effector domains of protein toxins typically of bacterial or plant origin.[41,42] The targeted cytotoxic protein approach is receiving increasing attention

for treatment of infectious diseases,[43] with pre-clinical antiviral activities demonstrated for each mode, i.e., ADCs,[44] radioimmunotherapy[45] and immunotoxins.[46-50]

In the present report, we describe a novel recombinant immunotoxin based on *Pseudomonas aeruginosa* exotoxin A (PE), in which the normal cell binding moiety is replaced by an antibody fragment against the KSHV gpK8.1A glycoprotein.[51] gpK8.1A is a 228 amino acid late lytic glycoprotein[52-54] displayed on KSHV virions;[55,56] it binds to heparin sulfate on the target cell surface,[57,58] thereby facilitating virus entry and consequent spread of infection. Antibodies against gpK8.1A are important serological markers for KSHV disease.[59] Antibody profiling for the various KSHV diseases has demonstrated that serum antibodies against gpK8.1A are 5-fold higher in patients with MCD compared with KS and PEL.[14] Importantly for the study reported here, the lytic phase of the KSHV infection cycle is associated with expression of gpK8.1A on the surface of at least a fraction of infected cells.[53-55,60] This viral glycoprotein therefore appears to be a potential target for an immunotoxin designed to kill lytically infected cells.

Results

Design and production of KSHV gpK8.1A-targeted recombinant immunotoxin. We designed an immunotoxin targeting gpK8.1A, based on the previously described murine mAb 4C3.[55] As shown schematically in **Figure 1**, the variable heavy and light chain cDNAs from the 4C3 hybridoma were isolated and used to generate a sequence encoding the corresponding single chain variable region fragment; this was linked to the cDNA encoding the PE38 moiety of PE, which lacks the normal N-terminal cell binding domain of the native toxin but contains the effector domains involved in translocation and cytotoxicity (ADP-ribosylation of elongation factor 2). The immunotoxin was expressed in *E. coli*, the inclusion body fraction was subjected to standard protocols of solubilization, denaturation and refolding, and the protein was purified by sequential ion exchange and size exclusion chromatography[61] (see Materials and Methods). This anti-gpK8.1A immunotoxin is herein designated 2014-PE38.

Target cell system for analyzing immunotoxin activity. As a cellular target to test the specific cytotoxic activity of 2014-PE38, we used a Vero cell line derivative (herein referred to as Vero-219) that is chronically infected with a recombinant KSHV (rKSHV.219) that carries two reporter genes, green fluorescent protein (GFP) driven by the constitutive elongation factor 1α strong cellular promoter and red fluorescent protein (RFP) linked to the KSHV lytic PAN promoter; GFP is expressed constitutively, i.e., in both latent and lytic phase, whereas RFP is expressed only upon induction into lytic phase.[62] Epifluorescence microscopy confirmed the expected result that without induction only a negligible fraction of Vero-219 cells expressed detectable RFP (**Fig. 2A** and top parts), whereas in induced cultures most of the cells (77–80% in >10 experiments) were RFP-positive.

We then examined expression of gpK8.1A on Vero-219 cells at 48 h post-induction by flow cytometry, staining with mAb 4C3 (or control IgG1) followed by Alexa Fluor 635-labeled second antibody. As shown in **Figure 2B**, K8.1 A staining was clearly

detected on a small subpopulation of induced cells; this pattern was not observed on uninduced cells (data not shown). The mean fluorescent intensity of the entire population of induced cells was 24.9 with mAb 4C3 compared with 8.0 with control IgG1. Additional perspective was gained by confocal microscopy analysis of the induced Vero-219 cells at 48 h post-induction (**Fig. 2C**). Beyond confirming the above finding of efficient induction into lytic phase as judged by most GFP-positive cells also expressing RFP, the images verify that gpK8.1A expression is highly variable within the induced population, with a subset of detectably positive cells as judged by surface (unpermeabilized) and intracellular (permeabilized) cell staining, but the majority of cells not detectably stained for gpK8.1A by this method.

Selective immunotoxin killing of induced KSHV-infected Vero-219 cells. As one test of the specific cytotoxic activity of 2014-PE38, we applied the direct killing assay based on cellular dehydrogenase-mediated reduction of the WST-8 tetrazolium salt to formazan.[63] The experiment shown in **Figure 3A** shows that the immunotoxin killed induced Vero-219 cells in dose-dependent fashion. Consistent with the data above indicating that in induced cultures most GFP positive cells also expressed RFP, most (75–90% in >10 experiments) of the induced cells were susceptible to killing by 2014-PE38. Specificity was verified by the minimal effect of an unrelated immunotoxin (BL22) that targets a B cell antigen not expressed on Vero-derived cells (**Fig. 3A**), and also by the lack of activity against uninduced Vero-219 cells (**Fig. 3B**). The killing was mediated by the cytotoxic action of the PE38 moiety, as evidenced by the ineffectiveness of 2014-PE38^{E553D} (**Fig. 3A**), which contains a single point mutation abolishing the ADP ribosylation activity of the PE38 moiety; this result confirms that the observed killing reflects the enzymatic activity of the immunotoxin and not simply an unrelated toxicity associated with binding of the antibody moiety to the target cells. The IC$_{50}$ for 2014-PE38 killing of induced Vero-219 cells was 146 ng/ml in **Figure 3A**, corresponding to 2.3 nM (range in 3 experiments: 139–170 ng/ml, mean 152 ng/ml; corresponding to 2.2–2.6 nM, mean 2.3 nM). These results suggest that despite the above-noted detection of gpK8.1A on only a small subpopulation of induced Vero-219 cells by immunofluorescence staining (**Fig. 2B and C**), the bulk of the cell population expressed sufficient surface levels of the viral glycoprotein to be sensitized for 2014-PE38-mediated killing.

Suppression of infectious KSHV production from Vero-219 cells by immunotoxin-mediated specific cytotoxicity. As a second test of the selective cytotoxic action of 2014-PE38, we examined the effect of the immunotoxin on infectious KSHV production, by titering culture supernatants of treated Vero-219 producer cells on fresh 293F target cells. The data in **Figure 4** demonstrate dose-dependent suppression of infectious virus release. Again specificity was documented by the lack of effect of the unrelated control immunotoxin BL22, and the dependence on immunotoxin-mediated killing of the producer cells was verified by the non-responsiveness to 2014-PE38^{E553D}. The IC$_{50}$ value of 124 ng/ml, corresponding to 1.9 nM (range in 4 experiments: 111–249 ng/ml, mean 150 ng/ml; corresponding to 1.7–3.8 nM, mean 2.3 nM) was comparable to that noted above in the direct killing assay.
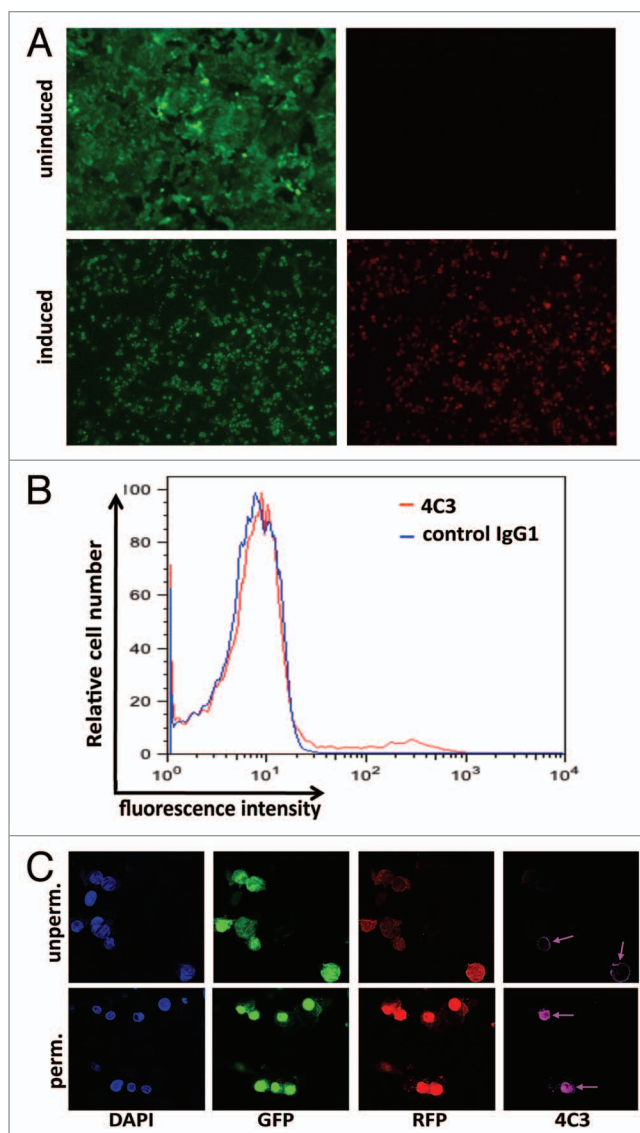

Figure 2. Characterization of Vero-219 cells. (A) The four parts are photomicrographs of Vero-219 cells containing rKSHV.219 showing detection of GFP under both induced (with Back50 + sodium butyrate stimulation, 24 h, lower parts) and uninduced (upper parts) conditions. (B) Flow cytometry analysis of K8.1A on the cell surface of induced Vero-219 cells by anti K8.1A mAb 4C3 (compared with isotype control antibody) at 48 h post-induction. (C) Confocal microscopic analysis of the staining of induced Vero-219 cells at 48 h post induction under un-permeabilized (upper parts) and permeabilized (lower parts) conditions.

Complementation of anti-KSHV activity by inhibition of viral DNA replication and targeted killing of infected cells. Nucleoside analogs that selectively inhibit viral DNA replication are the first line anti-herpesvirus drugs. Originally developed to treat herpes simplex virus infection, they also inhibit KSHV in vitro[60,64-66] and have displayed activity against KSHV-associated disease based on reductions in viral oropharyngeal shedding[67,68] and clinical relief of MCD symptoms.[34,35] At the outset, we considered three alternatives: (1) ganciclovir and the immunotoxin might display additive anti-KSHV activities; (2) they might
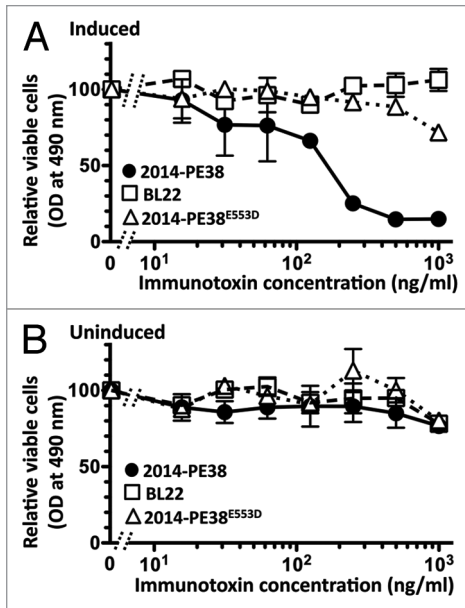
Figure 3. Specific killing of induced Vero-219 cells by 2014-PE38 as measured by the direct cytotoxicity assay. Induced (A) or uninduced (B) cells were treated for 72 h with the indicated concentrations of 2014-PE38 (solid circles) or negative control proteins BL22 (open squares) or 2014-PE38^{E553} (open triangles). Cell viability was measured by the WST-8 assay. Data points represent mean of duplicate samples; error bars represent standard deviation.
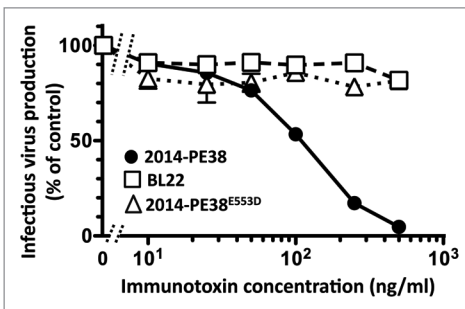
Figure 4. Specific killing of induced Vero-219 cells by 2014-PE38 as measured by the infectious virus release assay. Vero-219 cells were induced by BacK50 plus sodium butyrate and treated for 72 h with the indicated concentrations of 2014-PE38 (solid circles) or the control proteins BL22 (open squares) or 2014-PE38^{E553} (open triangles). The supernatants were harvested and assayed for infection of fresh 293 cells to measure the infectious KSHV-r129 produced from the activated cells. The amount of infectious virus produced from activated Vero-219 cells with no addition was set as 100%. Each data point represents the mean of duplicate culture wells with error bars as standard deviation.

potentiate one another through their differential mechanisms of inhibiting production of infectious virus during the lytic phase of KSHV replication; (3) ganciclovir might compromise immunotoxin activity by suppressing expression of the gpK8.1A target antigen.[60,66] We therefore examined the activities of each agent alone and in combination over wide concentration ranges. In the experiment shown in **Figure 5**, the individual IC$_{50}$ values were

249 ng/ml (corresponding to 3.8 nM) for 2014-PE38 and 1.15 μM for ganciclovir. When used in combination, each drug considerably enhanced the potency of the other (i.e., reciprocally reduced the IC$_{50}$ values). Thus, with ganciclovir at 1 μM (comparable to its intrinsic IC$_{50}$), the IC$_{50}$ of the immunotoxin was reduced by more than 5-fold (from 249 ng/ml to 43 ng/ml) (**Fig. 5A** and inset); conversely with 2014-PE38 at 125 ng/ml (less than its intrinsic IC$_{50}$), the IC$_{50}$ of ganciclovir was reduced more than 2-fold (from 1.15 μM to 0.48 μM) (**Fig. 2B** and inset). Thus there was no evidence for antagonism between these two agents that act by different mechanisms against the lytic phase; instead the opposite effect was observed, i.e., mutual potentiation of the inhibitory activities, beyond simple additivity.

Discussion

The data presented herein demonstrate that 2014-PE38 selectively kills KSHV-infected cells in dose-dependent fashion. The efficacy of this immunotoxin is demonstrated by the potent IC$_{50}$ values (ranging from 111–249 ng/ml) observed in assays of both direct target cell killing (**Fig. 3**) and suppression of infectious virus production (**Fig. 4**); moreover, its activity in the former assay was observed even under conditions of low surface expression of the gpK8.1A antigen on most of the target cells (**Fig. 2B and C**). This latter phenomenon has been observed with other PE-based immunotoxins; for example the anti-HIV immunotoxins sCD4(178)-PE40 [69] and 3B3-PE38 [70] both selectively kill productively HIV-1 infected primary macrophages despite the extremely low surface expression of the corresponding gp120 target antigen on the infected macrophage surface.[71] We note that in recent anti-cancer clinical trials of different PE-based recombinant immunotoxins, the mean plasma concentrations achieved at efficacious doses (at or below the maximum tolerated doses) exceeded the IC$_{50}$ values obtained here for 2014-PE38, e.g., 483 ng/ml for the anti-mesothelin immunotoxin SS1P[72] and >700 ng/ml for the anti-CD22 immunotoxin moxetumomab pasudotox.[73] That said, the median plasma half-lives of PE-based immunotoxins are relatively short (e.g., ~7–8 h for SS1P[72] and ~3 h for BL22,[74] the earlier version of moxetumomab pasudotox). Thus, it may be critical to enhance the potency of 2014-PE38, e.g., by affinity enhancement of the anti-K8.1A scFv moiety using phage display selection, as has been accomplished in generating the more potent moxetumomab pasudotox from its parent BL22.[75]

The 2014-PE38 immunotoxin may have particular therapeutic potential for treating MCD, the KSHV-mediated pathology most associated with the lytic phase of the viral life cycle, when gpK8.1A is expressed. This notion is supported by emerging clinical data for MCD treatment testing the effects of inhibitors of herpes virus replication such as ganciclovir and related agents. In one case study, ganciclovir was associated with reduction in the frequency of active MCD episodes,[34] although in another report cidofovir was found to be ineffective[76] despite its strong anti-KSHV activity in vitro. In a recent pilot study,[35] high-dose zidovudine plus valganciclovir produced significant clinical, biochemical and virologic responses in symptomatic

MCD patients; the results were interpreted as reflecting mainly (in addition to the possible direct anti-KSHV activity of valganciclovir) the ability of the products of the KSHV genes *ORF21* (encoding thymidine kinase) and *ORF36* (encoding a phosphotransferase) to activate the conversion of zidovudine and ganciclovir, respectively, into cytotoxic compounds. The efficacy was thus considered to be an example of "virus-activated cytotoxic therapy." Irrespective of the predominant mechanistic basis for the clinical benefits reported in that study, the present demonstration of selective killing of KSHV infected cells by 2014-PE38 suggests a potential role for this immunotoxin in treating MCD. Moreover, the mutually potentiating effects observed between 2014-PE38 and ganciclovir (**Fig. 5**) suggest that the combination might be particularly effective, and might achieve clinical benefits at lower doses of each agent, thereby minimizing toxic side effects. We have recently made similar arguments for another KSHV-directed immunotoxin targeting the viral gH glycoprotein.[77] Indeed in a murine herpes virus model, complementing activities between an immunotoxin and viral DNA replication inhibitors were observed both in vitro and in vivo.[78] Similarly, the robust synergistic activities between an anti-HIV immunotoxin and HAART drugs have been reported. Whereas CD4-PE40 alone or a reverse transcirptase inhibitor alone suppressed but did not eliminate HIV-1 from infected cell cultures,[79,80] the combination of the two agents completely "cured" the culture as assessed by eradication of infectious virus and elimination of viral DNA as determined by quantitative polymerase chain reaction analysis.[79] Pronounced complementation between anti-HIV immunotoxins and HAART drugs was also observed in an in vivo murine model.[81] These results highlight the special advantage of complementing drugs that inhibit the viral replication cycle with immunotoxins that kill already-infected cells.

The present findings should be viewed in the context of the general theme of targeted cytotoxic proteins, since major advances have been made in all three modes (i.e., ADCs, radioimmunotherapy, immunotoxins) discussed in the Introduction. In 1999, Ontak, a recombinant fusion protein consisting of IL2 linked to the translocation and cytotoxic domains of diphtheria toxin, became the first targeted cytotoxic protein to receive US Food and Drug Administration (FDA) approval (for treatment of cutaneous T cell lymphoma).[82] Impressive clinical results have since been obtained with other immunotoxins,[42] including PE-based[75] and diphtheria toxin-based[83] agents targeting hematologic cancers. The ADC gemtuzumab ozogamicin (Mylotarg®) received FDA approval in 2000 for treatment of acute myeloid leukemia, but was withdrawn due to lack of efficacy and toxicity concerns;[84] in subsequent years several new antibody-drug conjugates (e.g., trastuzumab emtansine, inotuzumab ozogamicin) have reached advanced stages of clinical development.[37,38,84] Two radioimmunotherapeutic proteins, ibritumomab tiuxetan (Zevalin®) and tositumomab-I131 (Bexxar®) are FDA-approved for treatment of certain types of lymphoma; several other radionuclide-conjugated antibodies are advancing in the clinical pipeline.[39,40]

In making general comparisons of these approaches, immunotoxins have the disadvantages of relatively short plasma half
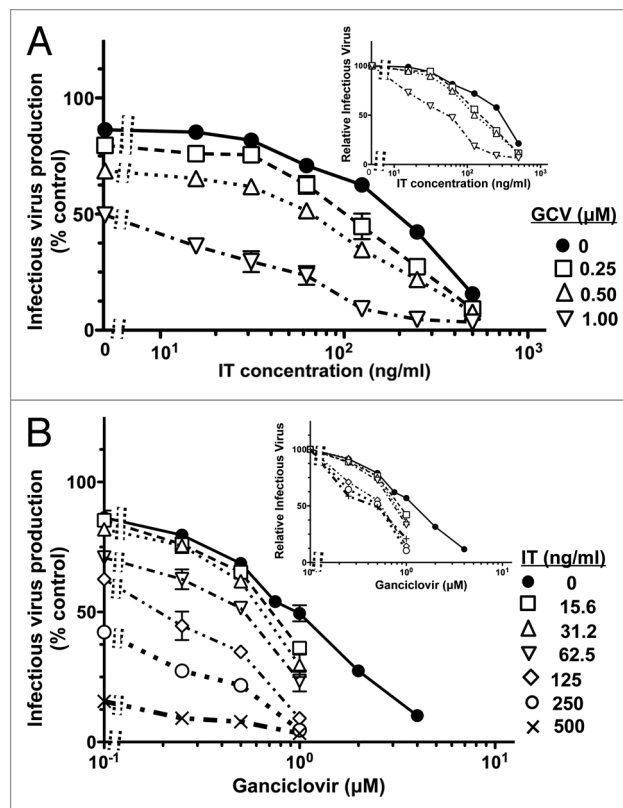
Figure 5. Mutual potentiation of 2014-PE38 and ganciclovir activities against KSHV infectious virus production from induced Vero-219 cells. Induced Vero-219 cells were treated for 72 h with combinations of 2014-PE38 and ganciclovir over the indicated concentration ranges for each agent. The amount of infectious virus produced in absence of immunotoxin or ganciclovir was set as 100%. Each data point represents the mean of duplicate culture wells with error bars indicating standard deviation. (A) Data are plotted as dose-response curves of 2014-PE38 in presence of the designated concentrations of ganciclovir; (B) Data are plotted as dose-response curves of ganciclovir in presence of the designated concentrations of 2014-PE38. In each part, the insets represent the same data sets, with the values obtained in absence of the agent on the X-axis normalized at 100% for each dose-response curve.

lives (generally ~2–4 h) and significant immunogenicity compared with the two alternative approaches employing intact immunoglobulins, which have intrinsically long half lives and can be humanized to reduce immunogenicity. However, marked advances have been achieved in minimizing these drawbacks and enhancing immunotoxin potential.[85,86] Efforts to increase the circulation time of these agents include the design of fusion proteins in which the immunotoxins are linked to Fabs[87] or to full-length immunoglobulins.[88] The immunogenicity problem is being addressed by identifying and eliminating B cell epitopes using site-directed mutagenesis[89] or by choosing human-derived proteins as the cytotoxic moieties.[90,91] In considering the application of any of these specific cell-killing modalities, treatment of viral infections has the advantage of targeting a viral-encoded antigen, thereby avoiding side-effects frequently encountered in cancer therapy owing to killing of normal cells expressing the targeted human antigen; on the other hand, a single virus-infected cell

can release thousands of infectious virions, thereby amplifying the impact of any infected cells that escape killing. Despite these complexities, the specific cytotoxic activity of the 2014-PE38 immunotoxin against K8.1A on KSHV-infected cells, coupled with its mutual potentiating activity when combined with a virus replication inhibitor, support the continued development of targeted cytotoxic protein approaches as components of treatment regimens for KSHV diseases, particularly MCD.

Materials and Methods

Construction and expression of plasmids encoding recombinant Immunotoxins. Total RNA was extracted from 4C3 hybridoma cells[55] by using the Qiagen RNeasy mini kit. Briefly, 5×10^6 cells were disrupted in 350 µl of buffer RLT containing β-mercaptoethanol and homogenized by loading onto a QIAshredder spin column and spinning for 2 min at a maximum speed in a micro centrifuge. 350 µl of 70% ethanol was added and transferred to an RNeasy spin column and centrifuged for 15 sec at 8,000 g. 700 µl of buffer RW1 was then added and spun for 15 sec at 8,000 g. 500 µl of buffer RPE was added and spun for 15 sec at 8,000 g. The RNeasy spin column was placed onto a new collection tube and RNA was eluted in 50 ul RNase-free water by centrifuging for 1 min at 8,000 g.

2.5 µg RNA and isotype-specific V_H hinge primer (MG1 hinge 5'-ACC ACA ATC CCT GGG CAC AAT TTT CT-3') or V_L hinge primer (MK-Edge 5'-CTC ATT CTT GTT GAA GCT CTT GAC AAT-3') were used to set up reaction for cDNA synthesis as described in the SMART RACE cDNA amplification kit (Clonetech, Paulo Alto, CA). The prepared cDNAs were used as templates in the 5' RACE PCR reactions with 10 pmol of isotype specific primers (MG1-PCR: 5'-AGG GGC CAG TGG ATA GAC AGA TGG GGG TGT-3', MK-PCR: 5'-GGA TGG TGG GAA GAT GGA TAC AGT TGG TGC AGC-3'). The PCR products were cloned into pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen). At least 20 clones for each chain were sequenced, analyzed and aligned according to the Ig BLAST program using the KABAT database (http://www.ncbi.nlm.nih.gov/igblast/).

The primers used to synthesize V_H and V_L fragments were designed according to the sequence alignment. The primers used to amplify the heavy chain Fv region were 5'4C3-VH20 (5'-AAA CAT ATG CAG ATC CAG TTG GTG CAG TCT-3') and 3'4C3-VH20-linker (5'-TCC AGA TCC GCC ACC ACC TGA TCC GCC TCC GCC TGA GGA GAC GGT GAC TGA GGT-3'). The primers used to amplify the light chain Fv region were 5'4C3-VL14-linker (5'-TCA GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC ATT GTG ATG ACA CAG TCT-3') and 3'4C3-VL14-HindIII (5'-GGA AGC TTT CCG TTT GAT TTC CAG CTT GGT-3'). The PCR products encoding the V_H and V_L domains of mAb 4C3 connected by a 15 amino acid linker $(Gly_4Ser)_3$ were generated by fusion PCR using the purified individual V_H and V_L PCR fragments through the primers 5'4C3-VH20 and 3'4C3-VL14-HindIII. The PCR product was cloned into pCR2.1 vector, double digested with EcoRV and HindIII and ligated to PE-toxin expression vector pTK21.8,[92] previously subjected to sequential single digestion with NdeI (polished with T4 Klenow fragment to generate a blunt end) and HindIII and alkaline-phosphatase treated. The resulting clone was named as 2014-PE38. We also constructed a clone 2014-PE38[E553D] encoding a negative control protein containing the E553D mutation in the PE38 domain that inactivates its ADP ribosylation activity.[93] The corresponding expression plasmids are designated pDC6 and pDC8, respectively. As a control we employed BL22-PE38, directed against CD22 antigen,[94] generously provided by Ira Pastan (NCI, NIH).

Expression and purification of immunotoxin. Immunotoxins were expressed and purified as described previously.[61,77] Briefly, the plasmids encoding the 2014-PE38 and 2014-PE38[E553D] were transformed into Max Efficiency DH5α *E. coli* BL21 (DE3) cells. Post overnight incubation at 37°C, the colonies were dislodged in 5 ml of super broth media and subsequently added onto a liter of pre-warmed super broth culture containing 20% glucose, 1.68 ml of 1 M $MgSO_4$ and ampicillin (100 µg/ml). Cells were incubated in shaking incubator 220 rpm at 37°C. When the $OD_{600 nm}$ was between 2.0 and 3.0, 500 ml culture was induced with 5 ml of 0.1 M IPTG and incubated at 37°C shaking for 90 min. The bacteria were harvested by centrifuging at 7,500 g at 4°C for 10 min. To prepare inclusion bodies, the bacterial pellet was resuspended in 160 ml of TES buffer (TES: 50 mM TRIS-HCl, pH 8.0, 20 mM EDTA, 100 mM NaCl) followed by treatment with 6.5 ml lysozyme and mixing using a Tissuemizer and incubated at room temperature for 30 min. 20 ml of 25% Triton-X100 was added, mixed with a Tissuemizer and incubated at RT for 30 min. The inclusion bodies were pelleted by centrifugation at 27,000 g for 50 min at 4°C and supernatant was discarded. The inclusion body pellet was washed thrice with TES-25% Triton-X100 and once without the triton-X100. The inclusion body pellet was denatured in 5 ml of denaturing buffer (6 M guanidine HCl, 100 mM TRIS-HCl, pH 8, 2 mM EDTA) using a Tissuemizer. The disulphide bonds in the denatured recombinant immunotoxin were reduced by addition of dithioerythritol powder (concentration 10 mg/ml) and incubation at room temperature overnight. The protein solution was centrifuged at 12,000 g for 10 min to remove the misfolded proteins and other debris in the pellet. Proteins were refolded by brisk stirring the supernatant in 100-fold excess volume of refolding buffer (100 mM TRIS-HCl, 1 mM EDTA, 0.5 M arginine, pH 9.5, chilled to 10°C) containing freshly added oxidized glutathione (551 mg/L). After mixing for 2–3 min, the solution was incubated at 10°C for 36–48 h. The recombinant immunotoxin preparation was finally dialyzed in dialysis buffer (20 mM TRIS-HCl, pH 7.4, 100 mM urea, chilled to 4°C) using dialysis tubing with a molecular weight cutoff of <50 kDa. The correctly folded immunotoxin was then passed through two ion exchange chromatography columns Q Sepharose and MonoQ (Amersham Pharmacia Biotech) and finally through size exclusion chromatography (TSK3000, TOSOH, Tokyo, Japan) to remove the impurities. Protein concentrations of the purified immunotoxins were determined by a protein assay (Bio-Rad) with bovine serum albumin as standard.

Cells and media. Vero-219 cells that harbor recombinant KSHV-219,[62] were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 5 ng/ml puromycin. 293F cells (derived from human embryonic kidney, Freestyle 293F subclone from Invitrogen) adapted to grow as adherent cultures[95] were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 1% non essential amino acids and 100 U/ml penicillin 100 μg/ml streptomycin.

Flow cytometry analyses. Vero-219 cells were infected with recombinant baculovirus BacK50 encoding the KSHV RTA trans activator[62] (M.O.I. 100) for 3 h at 37°C followed by aspiration of inoculums and addition of fresh media containing 1.25 mM sodium butyrate for overnight incubation. Post 24 h, cells were replenished with fresh media and incubated overnight. The cells were incubated with anti-gpK8.1A mAb 4C3 and IgG1 isotype control at a concentration of 5–10 μg/ml for 1 h at 4°C. The cells were washed with PBS thrice and incubated with secondary anti-mouse antibody conjugated with fluorescent dye Alexa 635 nm (1:500 dilution) for 30 min at 4°C, subsequently washed thrice with PBS, fixed with 2% PFA and run in a FACScalibur flow cytometer.

Confocal microscopy. 12 mm coverslips placed in 24-well culture plates were coated with 0.1% poly l-lysine, incubated overnight in a 37°C tissue culture incubator, washed thoroughly with deionized H_2O and seeded with Vero-219 at 100 x 10³/well. Vero-219 cells were infected with BacK50 (M.O.I. 100) for 3 h at 37°C followed by aspiration of inoculate and addition of fresh media containing 1.25 mM sodium butyrate for overnight incubation. Post 24 h, cells were replenished with fresh media and incubated overnight. The cells were either left untreated or fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton x 100 for 5 min, washed with PBS and stained with anti-gpK8.1A mAb 4C3 and mouse IgG control at a concentration of 5 μg/ml for 1 h at 4°C. Cells were washed thrice with PBS and incubated with secondary anti-mouse antibody conjugated with fluorescent dye 635 nm for 1 h at 4°C. Following three PBS washes, DAPI and mounting media were added and cells were viewed under confocal microscope SP2.

Direct cytotoxicity assay. Vero-219 were seeded in 96-well plates at 5 x 10⁴ cells/well and incubated overnight at 37°C, 5% CO_2. The cells were infected with BacK50 at M.O.I. 100 for 3 h at 37°C, washed with PBS once and replenished with complete medium supplemented with 1.25 mM sodium butyrate and different dilutions of immunotoxin (2-fold serial dilution starting from 2,000 ng/ml to 15.6 ng/ml, 100 μl total volume in each well) and incubated overnight. The supernatant was removed and cells were replenished with fresh media without sodium butyrate containing same immunotoxin dilutions and incubated for an additional 48 h at 37°C, 5% CO_2. Ten microliters of the Cell-Counting Kit-8 (CCK-8) solution (Dojindo Inc., Maryland) was added to the cells and incubated for 2 h at 37°C, 5% CO_2. The absorbance was measured at 450 nm using a microplate reader.

Virus infectivity assay. Vero-219 cells were seeded in 24-well plates at 1-2 x 10⁵ cells/well and incubated overnight. The cells were infected with BacK50 at M.O.I. 100 for 3 h at 37°C, washed with PBS once and replenished with complete medium supplemented with 1.25 mM sodium butyrate and different dilutions of immunotoxin (2-fold serial dilution starting from 2,000 ng/ml to 15.6 ng/ml, 500 μl total volume in each well) or ganciclovir (2-fold serial dilution starting from 4.0 μM to 0.25 μM in each well) and incubated overnight. The supernatants were removed and cells were replenished with fresh media without sodium butyrate containing the same immunotoxin dilutions and incubated for 48 h at 37°C, 5% CO_2. Fresh 293F cells were seeded (24 well plates, 2 x 10⁵ cells/well) and spin-inoculated with supernatants from the Vero-219 (1,500 g, 30 min) followed by incubation for 3 h at 37°C, 5% CO_2. After 3 h, the supernatants were aspirated and cells were replenished with fresh media and incubated for 48–72 h. The number of infected target cells was determined by flow cytometry; infectivity is expressed as percentage of target cells that scored GFP-positive, which indicated establishment of infection by KSHV.

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