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Rationally designed chemokine-based toxin targeting the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus infection in vivo

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The use of receptor–ligand interactions to direct toxins to kill diseased cells selectively has shown considerable promise for treatment of a number of cancers and, more recently, autoimmune disease. Here we move the fusion toxin protein (FTP) technology beyond cancer/autoimmune therapeutics to target the human viral pathogen, human cytomegalovirus (HCMV), on the basis of its expression of the 7TMD protein-coupled chemokine receptor US28. The virus origin of US28 provides an exceptional chemokine-binding profile with high selectivity and improved binding for the CX3CL1, CXCL1, and CXCR1 receptors, respectively.

Rationally designed chemokine-based toxin targeting high-affinity receptor interactions to direct toxins to kill diseased cells selectively has shown considerable promise for treatment of a number of cancers and, more recently, autoimmune disease. Here we move the fusion toxin protein (FTP) technology beyond cancer/autoimmune therapeutics to target the human viral pathogen, human cytomegalovirus (HCMV), on the basis of its expression of the 7TMD protein-coupled chemokine receptor US28. The virus origin of US28 provides an exceptional chemokine-binding profile with high selectivity and improved binding for the CX3CL1, CXCL1, and CXCR1 receptors, respectively.

**Significance**

All drugs currently used for the clinical treatment of human cytomegalovirus (HCMV) infection are associated with considerable adverse side effects and with the development of drug resistance that results in therapy failure. Here we describe a novel, rationally designed fusion toxin protein (FTP)-based strategy to target HCMV on the basis of its virally expressed G protein-coupled receptor (US28) and cognate chemokine ligand. G Protein-coupled receptors are expressed by a number of other clinically important viruses. We suggest that FTP-based molecules targeting virally expressed 7TM receptors may represent a new class of drugs amenable for development against complex viral pathogens.


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Conflict of interest statement: M.M.R. and T.N.K. declare a conflict of interest: US patent US8592554 B2 and EU patent PCT/DK2007/050082, owned by INAGEN ApS, have been filed for the use of immunotoxins for the treatment of diseases related to CMV infection. Freely available online through the PNAS open access option.

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cells through their expression of a virus-encoded seven-transmembrane (7TM) chemokine receptor, US28. A number of pharmacological and cellular properties of US28 suggest that this viral G protein-coupled receptor might be suited for targeting HCMV with a FTP-based strategy (Fig. 1 A and B). First, US28 binds a broad spectrum of chemokines as part of its immune-evasive function through chemokine scavenging. Importantly, US28 shows high selectivity and enhanced binding for the CXCL1 chemokine CXCL1 (24, 25). In contrast to most other human chemokines, CXCL1 consists of a chemokine domain, a mucin stalk, and a transmembrane domain (Fig. 1C) providing membrane anchorage of CXCL1 (26). The chemokine domain of CXCL1 has high affinity for US28 both after cleavage and when CXCL1 is attached to the mucin-like stalk extrusion from the chemokine domain (24, 25). Moreover, CXCL1 binds only a single human chemokine receptor, CXCR1, thus decreasing the potential for unwanted off-target effects of a CXCL1-based FTP strategy. Second, the majority of US28 is localized within endosomes, away from the cell surface (27). This distribution is a result of rapid, constitutive, ligand-independent receptor internalization (28) and is a feature suited for efficient intracellular delivery of FTP molecules. Based on the molecular characteristics of US28 and its defined ligand-binding profile, we designed an FTP consisting of the chemokine domain of CXCL1 and domains of PE (Fig. 1 C–E). The structural characteristics of CXCL1 suggest that the protein can sustain high-affinity binding to US28 when the C-terminally attached mucin-like stalk is replaced by other proteins, e.g., the PE domains associated with translocation (domain II) and cytotoxicity (domains Ib and III). PE is a very potent toxin capable of killing cells via its adenosine diphosphate-ribosylation domain that modifies elongation factor 2, leading to the arrest of protein synthesis and the initiation of apoptosis (29, 30).

The aim of the current study was to explore an FTP-based approach using an engineered chemokine fused to a toxin to target a human viral pathogen, HCMV, on the basis of its expression of the constitutively internalizing 7TM receptor, US28 (Fig. 1B).

### Results

**CXCL1-FTP Has Increased Binding Affinity for HCMV US28 Compared with CXCR1.** First, to determine whether a toxin could be targeted to HCMV-infected cells via cell surface-expressed US28, we created a fusion protein, CXCL1-FTP (Fig. 1E). The CD91 receptor binding of PE (domain I) was removed to limit entry to only US28-expressing cells. Consistent with CXCL1’s high affinity for US28, which is unaltered whether the chemokine domain is alone or is attached to the mucin stalk (24, 25), the replacement of the mucin-like stalk with three PE domains to create the CXCL1-FTP construct did not prevent binding to US28 (Fig. 1F). Importantly, CXCL1-FTP maintained high affinity for US28 with a less than fourfold decrease in affinity compared with the WT CXCL1 chemokine [0.19 nM (log IC50 = −9.7 ± 0.52) and 0.67 nM (log IC50 = −9.2 ± 0.08)], respectively, using 125I-I-CCL2 as the radioligand] (Fig. 1F). In contrast, CXCL1-FTP’s affinity for CXCR1 was 145-fold less than that of CXCL1 [IC50 = 53 nM (log IC50 = −7.3 ± 0.15) and 0.37 nM (log IC50 = −9.4 ± 0.12), respectively, using 125I-CXCL1-FTP as the radioligand] (Fig. 1G). Together, these results show that fusion of CXCL1 to PE domains results in a recombinant FTP molecule (CXCL1-FTP) that has a selectivity profile that favors binding to US28 with 80-fold higher affinity for US28 than for CXCR1 (Fig. 1 F and G).

**CXCL1-FTP Preferentially Kills Cells Expressing US28.** To assess the capacity of CXCL1-FTP to deliver the chemokine-coupled PE toxin payload intracellularly, we measured in vitro cell-killing activity in cells expressing either US28 or CXCR1 under the control of a tetracycline-inducible promoter. As shown in Fig. 1H, CXCL1-FTP effectively killed US28-expressing cells, with 60% cell killing at 0.1 nM [IC50 = 0.03 nM (log IC50 = −10.5 ± 0.37)]. Consistent with its decreased affinity for CXCR1, 0.1 nM CXCL1-FTP caused a lower degree of cell killing (10%) in CXCR1-expressing cells but retained high potency [IC50 = 0.39 nM (log IC50 = −9.4 ± 0.48)]. In contrast, cells with no receptor expression (noninduced cells) showed high viability even at 1 nM CXCL1-FTP. The observed differences in maximum killing of cells expressing US28 compared with cells expressing CXCR1 (70% versus 40%) may be caused by differences in receptor cellular distribution profiles and/or recycling properties (27). Together, these results show that US28-expressing cells are killed efficiently by CXCL1-FTP. The reduced killing of cells expressing CXCR1 compared with cells expressing US28 is consistent with CXCL1-FTP’s higher affinity for US28 than for CXCR1 (Fig. 1 F and G).

**CXCL1-FTP Kills HCMV-Infected Cells With Potency Surpassing That of GCV.** To test the antiviral efficacy and potency of CXCL1-FTP within the context of infection, CXCL1-FTP was added to HCMV (Towne strain)-infected human foreskin fibroblasts (HFFs). In single-dose experiments, CXCL1-FTP completely inhibited the...
release of virus particles, with a potency surpassing by 104-fold that of the current first-line HCMV drug, GCV [IC50 = 1.9 nM (log IC50 = −8.7 ± 0.08) and 18 μM (log IC50 = −4.7 ± 0.11), respectively] (Fig. 2A). In repeated-dose experiments in HCMV-infected cells, cultures treated with 0.3 nM (log concentration −9.5) or higher doses of CX3CL1-FTP developed a fully confluent cell monolayer by 8 days postinfection indicating complete control of virus replication (Fig. 2B). Consistent with its lower potency, 30 μM (log concentration −4.5) GCV was required to achieve a similar antiviral effect. HFFs are in principle susceptible to CX3CL1-FTP, because detectable CX3CR1 expression levels have been published in these cells (31). However, because CX3CL1-FTP’s affinity for CX3CR1 is 80-fold lower than its affinity for US28, a much higher concentration of the FTP would be required to kill CX3CR1-expressing cells (Fig. 1 F and G). Collectively, these results show that, in the context of HCMV infection, CX3CL1-FTP has high potency for killing US28-expressing cells.

The Antiviral Activity of CX3CL1-FTP Is Caused by Direct Killing of HCMV-Infected Cells. To investigate whether the antiviral activity of CX3CL1-FTP was a consequence of direct and selective killing of HCMV-infected cells, we studied the inhibition of viral replication in human fetal lung fibroblasts (MRC-5) infected with the HCMV Toledo01U strain that contains a luciferase reporter gene (32). In this modified strain, luciferase activity can be monitored as a direct measure of viral replication (33). Compared with the laboratory Towne strain, Toledo also is regarded as having characteristics more similar to clinical HCMV isolates (34). In single-dose experiments, CX3CL1-FTP inhibited luciferase activity efficiently, with a potency 104-fold higher than that of GCV [IC50 = 1.7 nM (log IC50 = −8.8 ± 0.18) and >2 μM (log IC50 higher than −2.7), respectively] (Fig. 2C), independent of the multiplicity of infection (MOI) (Fig. S1). In repeated-dose experiments, treatment of infected cells with 1 nM CX3CL1-FTP inhibited luciferase activity below the detection level (Fig. 2D). This robust level of viral control could not be obtained with GCV, where an increase in luciferase activity began at 5 days postinfection, even when using a 105-fold higher concentration (0.1 mM) (Fig. 2D). CX3CL1-FTP also was compared with another currently used anti-HCMV therapeutic, Klovig (human normal Ig administered i.v.) and again showed superior efficacy and viral control in both single- and repeated-dose experiments (Fig. S2).

The Antiviral Activity of CX3CL1-FTP Is Effective Against GCV-Resistant HCMV Clinical Isolates. To assess further the clinical potential of CX3CL1-FTP, antiviral activity was evaluated using a GCV-resistant clinical isolate, HCMV-SV2. This strain remained highly sensitive to CX3CL1-FTP but, as expected, was relatively unaffected by GCV treatment, with the potency of CX3CL1-FTP being 4 × 104-fold greater than the potency of GCV in single-dose experiments (Fig. 2E). In repeated-dose experiments, 1 nM [log concentration −9] CX3CL1-FTP resulted in complete virus control after 6 days of treatment (Fig. 2F). Speed of action is an important feature of an antiviral drug, particularly in treating immune-compromised transplant patients for whom the time of treatment can determine the difference between successful organ engraftment or rejection (35). Together, our results show that CX3CL1-FTP is a highly potent inhibitor of both laboratory and clinical strains of HCMV. Moreover, the virocidal mechanism (i.e., killing of infected cells) of CX3CL1-FTP appears to be intrinsically faster acting, at least in vitro, than the virostatic mode of action of GCV.

CX3CL1-FTP Resistance After Long-Term Treatment in Vitro. To investigate whether treatment with CX3CL1-FTP could lead to resistance, long-term treatment was initiated. The results show that CX3CL1-FTP is not in itself unreceptive to the development of resistance in vitro. A CX3CL1-FTP-resistant HCMV TOWNE strain appeared after 3 weeks of selection under CX3CL1-FTP. The causative mutation was a single G-to-A mutation at position 774 within the US28 gene, resulting in a premature stop codon at amino acid residue 258 in the third extracellular loop of US28 (Fig. S3A). This truncated variant of US28 (1-257) was expressed at the cell surface, although to a lower degree than US28 WT (Fig. S3B). However, competition binding experiments with radio-labeled CX3CL1 and CCL2 revealed impaired chemokine binding for US28 (1-257), and chemotaxis analyses confirmed that the US28 (1-257) had lost its ability to bind chemokines, because it failed to adhere to and migrate on surfaces presenting FL CX3CL1 (Fig. S3 C–F). Because US28-mediated chemokine scavenging and migration are believed to be critical for immune evasion (36, 37), an inability of US28 to bind chemokines likely would affect the fitness of F49A-FTP-resistant variants and possibly would impact their capacity to persist in vivo.

The Selectivity of Rationally Designed CX3CL1 Molecules for US28 Is Superior to That of CX3CR1. Because CX3CL1-FTP also killed cells expressing the human chemokine receptor CX3CR1 (albeit much less efficiently; Fig. 1F), we used a rational design strategy to enhance further the selectivity of the FTP molecule toward US28. Based on our knowledge of chemokine domains important for receptor recognition and action (21, 22), we designed and expressed 35 different CX3CL1 variants (numbered 1 through 35) with mutations in the CX3CL1 chemokine component and measured their affinity for US28 and CX3CR1 (Fig. 3, Fig. S1, and Table S1). Because CC-chemokines and CX3CL1 are known to bind to US28 through distinct mechanisms (24, 25), we used two radioligands (125I-CCL2

Fig. 2. Anti-HCMV activity of CX3CL1-FTP. (A) Inhibition of virus particle release measured as pfu from HFFs infected with HCMV Towne at a concentration corresponding to one virus particle per 10 cells (MOI of 0.1) and treated with a single dose of CX3CL1-FTP (black circles) or GCV (open squares). (B) HFFs 8 d after infection with HCMV Towne (MOI of 0.1). The cells were treated with CX3CL1-FTP or GCV at the indicated concentrations (10−8−10−12 M (−9, −9.5, −10) and 10−4−10−3 M (−4, −4.5, −5), respectively) on days 1, 3, 5, and 8 postinfection. (C) Inhibition of virus replication measured by luciferase activity in human lung fibroblasts (MRC-5) infected with HCMV Toledo01U (MOI of 0.1) and treated with a single dose of CX3CL1-FTP or GCV at the indicated concentrations. RU, relative luminescence units. (D) Effect of repeated drug treatments on virus replication in MRC-5 cells infected with Toledo01U (MOI of 0.1). The cells were treated on days 1, 3, 5, and 6 postinfection with CX3CL1-FTP (10−9 M), GCV (10−8 M), or buffer (negative controls), and virus replication was measured by luminescence. (E) Inhibition of virus particle release from MRC-5 cells infected with the GCV-resistant HCMV-SV2 (MOI of 0.01) and treated twice (on days 2 and 3 after infection) with either CX3CL1-FTP or GCV at the indicated concentrations. (F) MRC-5 cells 6 d after infection with the GCV-resistant HCMV-SV2 (MOI of 0.01) and treated twice (on days 2 and 3) with CX3CL1-FTP (10−8 M) (Right) or buffer (Left). Error bars in A, C, D, and E indicate SEM for three independent biological replicates.

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and the F49A mutation results in an 18-fold decrease in affinity for US28 (Fig. 3). \( \text{IC}_{50} \) for US28 was 10 nM (log \( \text{IC}_{50} \) = −1.0 ± 0.13) and 0.28 mM (log \( \text{IC}_{50} \) = −3.6 ± 0.22), respectively (Fig. 4B), and the efficacy of repeated treatments with 1 nM F49A-FTP also was superior to that of 0.1 mM GCV (Fig. 4C).

In a final series of experiments, the efficacy of F49A-FTP was assessed in vivo in an SCID-hu mouse model, a state-of-the-art model that enables the replication of clinical HCMV isolates to be analyzed in vivo (38). SCID-hu mice were implanted with human fetal thymus/liver tissue under the kidney capsule. After engraftment the implants were infected with the HCMV ToledoLuc strain by direct inoculation. Luciferase activity was serially monitored as an indicator of in vivo virus replication in real time (i.e., without killing the mice) (Fig. 4 D and E). In total, implants in 17 SCID-hu mice were surgically inoculated with ToledoLuc, and these mice were divided into the following experimental treatment groups: 0.4 mg/kg F49A-FTP (\( n = 5 \)), 8 mg/kg GCV (\( n = 5 \)), or mock treatment (\( n = 7 \)). Five additional mock-treated SCID-hu mice were included as noninfected controls. The GCV dose was based on previous studies using a similar model and on the clinical recommendations of 5 mg kg\(^{-1}\)·d\(^{-1}\) in patients (33, 39). One mock-treated infected mouse died at day 12 postinfection, but no mice in any of the other treatment groups died during the course of the experiment. The lack of gross toxic effects in uninfected and infected mice suggests that F49A-FTP has minimal toxicity in vivo.
mice indicates that there is no binding with subsequent receptor internalization of F49A-FTP to mouse CX3CR1. Mock-treated HCMV-infected mice developed significant bioluminescence signals over the 14-day period of assessment, indicating highly productive HCMV replication. In contrast, F49A-FTP–treated mice showed only a transient, weak signal on days 4–6 post-infection which then dropped to background levels (Fig. 4 D and E). At peak luciferase activity (day 8), the bioluminescence signal of F49A-FTP–treated mice was 10-fold lower than that of GCV–treated mice. In summary, these results in the SCID-hu mouse model identify F49A-FTP as a highly effective inhibitor of virus replication in vivo that appears to be far superior to GCV.

Discussion

In the present study we show that an approach based on a rationally designed FTP molecule using a synthetic CX3CL1 chemokine engineered for ultra-high affinity and selectivity for the US28 receptor can be used to develop a potent antiviral agent for HCMV. The efficacy of the derivative FTP-based antiviral drugs was superior to that of the anti-HCMV drugs GCV and Kiovig. To date, FTP-based strategies generally have been studied for the treatment of cancer and autoimmune disease. Our study also underscores the strength of rational drug design–based approaches both in the selection of appropriate receptor–ligand interactions and in further molecular refinement to reduce undesirable FTP-binding characteristics. This strategy resulted in the development of a second-generation FTP, F49A-FTP, that had a higher selectivity profile toward US28 and that controlled HCMV infection in vivo at levels surpassing GCV. The high efficacy of F49A-FTP demonstrates the potential of this molecule for further development as an anti-HCMV therapeutic drug. Moreover, the recently reported structure of the US28–CX3CL1 complex providing the structural basis for chemokine recognition of US28 will further increase the possibility of developing this FTP into a drug with a superior therapeutic index. F49A-FTP’s novel mechanism of action may increase treatment options of patients for whom GCV is not a possibility because of toxicity or the development of viral resistance to DNA polymerase inhibitors (18, 19). Combination therapy of F49A-FTP–based drugs with first-line agents also may allow lower levels to be used clinically, thereby reducing toxicity side effects. In contrast to viru-ostatic DNA polymerase inhibitors such as GCV, FTPs inhibit HCMV by a virocidal mechanism of direct killing of infected cells before the release of virus progeny. This difference in the mecha-nism of virus control may be responsible for the more complete level of HCMV inhibition observed for F49A-FTP as compared with GCV. PE is known to induce apoptosis efficiently after delivery into the cell (30). Importantly, PE–mediated apoptosis is not blocked by the HCMV-encoded inhibitor of caspase 8 that inhibits programmed cell death pathways (40, 41). Many questions clearly remain, including the capacity for development of F49A-FTP resistance in vivo. Fusion to toxins of even greater biocidal activity may further enhance the FTP antiviral activity and thereby decrease the possibility of the development of drug resistance. Questions regarding immunogenicity associated with F49A-FTP also will need to be addressed, but thus far FTP–directed immune responses do not appear to affect the efficacy of FTPs substantially when used in cancer (42). Antibodies directed against a PE–based FTP in a mouse pulmonary fibrosis (autoimmunity disease) model were shown not to reduce the biological activity (43).

The crystal structure of the human CX3CL1 chemokine domain bound to US28 serves as a model for viral chemokine 7TM-ligand complexes. Viral G protein-coupled receptors are expressed by a number of viruses in addition to HCMV, and some of these are anticipated to function in a manner similar to US28. Rationally designed FTP-based strategies targeting virus–expressed receptors may provide promising drug-target pairs not only for anti-HCMV therapy but also for other viruses that express similar constitutively internalized receptors with known ligand-binding profiles.

Methods

Radioligand Competition Binding Assays. Stable inducible clones of US28/ CX3CR1-HEK293 cells were grown as previously reported (44). Briefly, the cells were seeded at 10,000 cells per well in poly–lysine (Invitrogen)–coated 96-well plates (Nunc). One day after seeding, US28 and CX3CR1 expression was induced by tetracycline (3.6 ng/mL and 5 ng/mL, respectively) to obtain 5–10% specific binding. Competition binding studies were performed in triplicate as described previously (45). Briefly, 1 μM of polyclonal serum were incubated for 3 h at 4 °C with 32 pM of the iodinated chemokine plus 6 nM of the unlabeled ligand in 125 μL buffer with 50 mM Hepes (Invitrogen) and 0.5% (wt/vol) BSA (Sigma). Afterward cells at 4 °C were washed twice with 200 μL 50 mM Hepes buffer supplemented with 0.5% (wt/vol) BSA and 0.5 M NaCl. Data were collected using a Gamma-counter.

In Vitro Killing Activity. Stable inducible clones of US28/CX3CR1-HEK293 cells were seeded at 5,500 cells per well in Poly–lysine–coated 48-well tissue-culture plates (Nunc) in 500 μL DMEM (Invitrogen) per well and were grown at 37 °C. Rhesus cytotoxicity receptor expression was induced 24 h after seeding using 0.25 μg/mL (US28) and 0.125 μg/mL (CX3CR1) tetracycline. The different concentrations of the indicated FTP (0.1 μM–1 μM) and buffer (mock treatment) were added 1 d after receptor induction in a final volume of 300 μL DMEM and were incubated for 24 h at 37 °C. To estimate cell viability, the cells were incubated with AlamarBlue (Invitrogen) in PBS (5 mg/mL), 300 μL per well, for 4 h at 37 °C. Data were collected using a Synergy HT plate reader.

Antiviral Activity. The antiviral effect of the immunomodulins was tested on the HCMV strains Towne and ToledoCR1 on the GCV-resistant clinical isolate HCMV-SV2. The impact on the release of infectious virus particles from HFFs (ATCC CRL-1634) was measured using the HCMV Towne strain. For single-dose experiments HFFs (70% confluent) were seeded in 24-well tissue-culture plates (Nunc) and were infected with HCMV Towne (ATCC VR-977) at an MOI of 0.1 (corresponding to one virus particle per 10 cells). The infection was allowed to proceed under exposure of a single dose of the respective FTP (1 μM–10 μM) or GCV (Sigma) (0.1 mM–1 nM) until the infected untreated cells reached 100% cytopathic effect. Virus titers in supernatants were measured by plaque assay. For repeated-dose experiments HFFs (120,000 cells per well) were seeded in six-well tissue-culture plates and were infected with HCMV Towne at an MOI of 0.01. PBS (100 μL) containing FTP, GCV, or control buffer was added every second day, and virus infection was documented by photographs.

The intracellular replication was measured using HCMV ToledoCR1 in MRC-5 human fetal lung fibroblasts (ATCC CCL-171). The recombinant HCMV ToledoCR1 virus was reconstituted from the HCMV BAC (28), and the expression of the luciferase gene in the viral genome was used to quantitate the magnitude of reverse transcription. For single-dose experiments MRC-5 cells (8,000 cells per well) were seeded in 96-well tissue-culture plates (Nunc) and were infected with ToledoCR1 at an MOI of 0.1. After the addition of a single dose of FTP (1 μM–10 μM), GCV (Sigma) (0.1 mM–1 nM), or Kiovig (Baxter) (68 nM–21 μM), the infection was allowed to proceed for 4 d; then the medium was replaced every third day and the cells were given growth medium with 0.7-Luciferin at a final concentration of 150 g/mL. The luciferase activity was measured on days 1, 3, 6, 8, and 10 postinfection using the Synergy HT plate reader.

Activity on a GCV-Resistant Clinical Isolate. MRC-5 cells (10,000 cells per well) were seeded in 24-well tissue-culture plates (Nunc) and were infected with the HCMV-SV2 strain (46) (kindly provided by Sebastian Voigt of the Robert Koch Institute, Berlin) at an MOI of 0.1. After 2 h of incubation the inoculum was replaced with 1 mL of a methylcellulose overlay medium, and the cells were treated with different concentrations of CX3CL1-FTP, GCV, or buffer (mock treatment) at 2 and 3 d postinfection. Cultures were incubated…
for 9 d, and the plaques were evaluated visually. The progress of the HCMV-52V infection in MRC-5 cells (MOI of 0.1) after treatment with 1 nM of CxCL1-FITC and buffer (mock treatment) was visualized photographically at 6 d postinfection.

In Vivo Studies. Toledo01-084 was diluted to a titer of 2.5 × 105 pfu with PBS and was kept on ice until use. Thirty male homozygous C.B-17 SCID/SCID mice were surgically implanted with a human fetal thymus/liver sandwich-like structure under the kidney capsule, as previously described (33). Three months after implantation, the SCID-hu mice were anesthetized, and implanted parabdominal and intraperitoneal injections were performed with 20 μL of Toledo01-084 (5 × 10^6 PFU). Luciferase activity was measured using an in vivo imaging system by injecting o-luciferin substrate i.p., as previously described (32, 33). The infected animals were treated by i.p. injection with buffer (mock), GCV (8 mg/kg), or F49A-FTP (0.4 mg/kg) on days 1, 3, and 5 postinfection. Bio-luminescent measurements were performed every 48 h. All animals were included in the analysis, and animal groups were not blinded. Human fetal tissues were obtained from Advanced Bioscience Resources with informed consent according to local, state, and federal regulations. Animal work was done under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (47), and the protocol was approved by the Rutgers-New Jersey Medical School Institutional Animal Care and Use Committee.

Molecular Modeling. Mutations of CxCL1 Phe46 were modeled on the 2.9-Å structure of CxCL1 bound to US28 (Protein Data Bank (PDB) ID code 4XT1) using PyMOL v1.7.4.0 (Schrodinger, LLC). For each mutation, the most probable rotamer that did not produce clashes was chosen. Structure figures were prepared with PyMOL.

Statistical Analysis. Data analyses were performed using Prism v6.01. Data are expressed as means ± SEM.

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