Distinct roles of extracellular domains in the epstein-barr virus-encoded BILF1 receptor for signaling and major histocompatibility complex class I downregulation

The Epstein-Barr virus (EBV) BILF1 gene encodes a constitutively active G protein-coupled receptor (GPCR) that downregulates major histocompatibility complex (MHC) class I and induces signaling-dependent tumorigenesis. Different BILF1 homologs display highly conserved extracellular loops (ECLs) including the conserved cysteine residues involved in disulfide bridges present in class A GPCRs (GPCR bridge between transmembrane helix 3 [TM-3] and ECL-2) and in chemokine receptors (CKR bridge between the N terminus and ECL-3). In order to investigate the roles of the conserved residues in the receptor functions, 25 mutations were created in the extracellular domains. Luciferase reporter assays and flow cytometry were used to investigate the G protein signaling and MHC class I downregulation in HEK293 cells. We find that the cysteine residues involved in the GPCR bridge are important for both signaling and MHC class I downregulation, whereas the cysteine residues in the N terminus and ECL-3 are dispensable for signaling but important for MHC class I downregulation. Multiple conserved residues in the extracellular regions are important for the receptor-induced MHC class I downregulation, but not for signaling, indicating distinct structural requirements for these two functions. In an engineered receptor containing a binding site for Zn\(^{2+}\) ions in a complex with an aromatic chelator (phenanthroline or bipyridine), a ligand-driven inhibition of both the receptor signaling and MHC class I downregulation was observed. Taken together, this suggests that distinct regions in EBV-BILF1 can be pharmacologically targeted to inhibit the signaling-mediated tumorigenesis and interfere with the MHC class I downregulation. IMPORTANCE G protein-coupled receptors constitute the largest family of membrane proteins. As targets of >30% of the FDA-approved drugs, they are valuable for drug discovery. The receptor is composed of seven membrane-spanning helices and intracellular and extracellular domains. BILF1 is a receptor encoded by Epstein-Barr virus (EBV), which evades the host immune system by various strategies. BILF1 facilitates the virus immune evasion by downregulating MHC class I and is capable of inducing signaling-mediated tumorigenesis. BILF1 homologs from primate viruses show highly conserved extracellular domains. Here, we show that conserved residues in the extracellular domains of EBV-BILF1 are important for downregulating MHC class I and that the receptor signaling and immune evasion can be inhibited by drug-like small molecules. This suggests that BILF1 could be a target to inhibit the signaling-mediated tumorigenesis and interfere with the MHC class I downregulation, thereby facilitating virus recognition by the immune system.
Novel Chemokine-Based Immunotoxins for Potent and Selective Targeting of Cytomegalovirus Infected Cells

Immunotoxins as antiviral therapeutics are largely unexplored but have promising prospective due to their high selectivity potential and their unparalleled efficiency. One recent example targeted the virus-encoded G protein-coupled receptor US28 as a strategy for specific and efficient treatment of human cytomegalovirus (HCMV) infections. US28 is expressed on virus-infected cells and scavenge chemokines by rapid internalization. The chemokine-based fusion-toxin protein (FTP) consisted of a variant (F49A) of CX3CL1 specifically targeting US28 linked to the catalytic domain of Pseudomonas exotoxin A (PE). Here, we systematically seek to improve F49A-FTP by modifications in its three structural domains; we
generated variants with (1) altered chemokine sequence (K14A, F49L, and F49E), (2) shortened and elongated linker region, and (3) modified toxin domain. Only F49L-FTP displayed higher selectivity in its binding to US28 versus CX3CR1, the endogenous receptor for CX3CL1, but this was not matched by a more selective killing of US28-expressing cells. A longer linker and different toxin variants decreased US28 affinity and selective killing. Thereby, F49A-FTP represents the best candidate for HCMV treatment. Many viruses encode internalizing receptors suggesting that not only HCMV but also, for instance, Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus may be targeted by FTPs.
Targeting the latent cytomegalovirus reservoir with an antiviral fusion toxin protein

Reactivation of human cytomegalovirus (HCMV) in transplant recipients can cause life-threatening disease. Consequently, for transplant recipients, killing latently infected cells could have far-reaching clinical benefits. In vivo, myeloid cells and their progenitors are an important site of HCMV latency, and one viral gene expressed by latently infected myeloid cells is US28. This viral gene encodes a cell surface G protein-coupled receptor (GPCR) that binds chemokines, triggering its endocytosis. We show that the expression of US28 on the surface of latently infected cells allows monocytes and their progenitor CD34+ cells to be targeted and killed by F49A-FTP, a highly specific fusion toxin protein that binds this viral GPCR. As expected, this specific targeting of latently infected cells by F49A-FTP also robustly reduces virus reactivation in vitro. Consequently, such specific fusion toxin proteins could form the basis of a therapeutic strategy for eliminating latently infected cells before haematopoietic stem cell transplantation.

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The future of antiviral immunotoxins

There is a constant need for new therapeutic interventions in a wide range of infectious diseases. Over the past few years, the immunotoxins have entered the stage as promising antiviral treatments. Immunotoxins have been extensively explored in cancer treatment and have achieved FDA approval in several cases. Indeed, the design of new anticancer immunotoxins is a rapidly developing field. However, at present, several immunotoxins have been developed targeting a variety of different viruses with high specificity and efficacy. Rather than blocking a viral or cellular pathway needed for virus replication and dissemination, immunotoxins exert their effect by killing and eradicating the pool of infected cells. By targeting a virus-encoded target molecule, it is possible to obtain superior selectivity and drastically limit the side effects, which is an immunotoxin-related challenge that has hindered the success of immunotoxins in cancer treatment. Therefore, it seems beneficial to use immunotoxins for the treatment of virus infections. One recent example showed that targeting of virus-encoded 7 transmembrane (7TM) receptors by immunotoxins could be a future strategy for designing ultraspecific antiviral treatment, ensuring efficient internalization and hence efficient eradication of the pool of infected cells, both in vitro and in vivo. In this review, we provide an overview of the mechanisms of action of immunotoxins and highlight the advantages of immunotoxins as future anti-viral therapies.
Rationally designed chemokine-based toxin targeting the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus infection in vivo

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 7TM G protein-coupled chemokine receptor US28. The virus origin of US28 provides an exceptional chemokine-binding profile with high selectivity and improved binding for the CX3C chemokine, CX3CL1. Moreover, US28 is constitutively internalizing by nature, providing highly effective FTP delivery. We designed a synthetic CX3CL1 variant engineered to have ultra-high affinity for US28 and greater specificity for US28 than the natural sole receptor for CX3CL1, CX3CR1, and we fused the synthetic variant with the cytotoxic domain of Pseudomonas Exotoxin A. This novel strategy of a rationally designed FTP provided unparalleled anti-HCMV efficacy and potency in vitro and in vivo.
Human cytomegalovirus chemokine receptor US28 induces migration of cells on a CX3CL1-presenting surface.

Human cytomegalovirus (HCMV)-encoded G protein-coupled-receptor US28 is believed to participate in virus dissemination through modulation of cell migration and immune evasion. US28 binds different CC chemokines and the CX3C chemokine CX3CL1. Membrane-anchored CX3CL1 is expressed by immune-activated endothelial cells, causing redirection of CX3CR1-expressing leukocytes in the blood to sites of infection. Here, we used stable transfected cell lines to examine how US28 expression affects cell migration on immobilized full-length CX3CL1, to model how HCMV-infected leukocytes interact with inflamed endothelium. We observed that US28-expressing cells migrated more than CX3CR1-expressing cells when adhering to immobilized CX3CL1. US28-induced migration was G protein-signalling dependent and was blocked by the phospholipase Cβ inhibitor U73122 and the intracellular calcium chelator BAPTA-AM. In addition, migration was inhibited in a dose-dependent manner by competition from CCL2 and CCL5, whereas CCL3 had little effect. Instead of migrating, CX3CR1-expressing cells performed 'dancing-on-the-spot' movements, demonstrating that anchored CX3CL1 acts as a strong tether for these cells. At low receptor expression levels, however, no significant difference in migration potential was observed when comparing the migration of CX3CR1- and US28-expressing cells. Thus, these data showed that, in contrast to CX3CR1, which promotes efficient cell capture upon binding to anchored CX3CL1, US28 acts to increase the migration of cells upon binding to the same ligand. Overall, this indicates that infected cells probably move more than uninfected cells in inflamed tissues with high CX3CL1 expression, with soluble chemokines affecting the final migration.

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Cell transformation mediated by the Epstein-Barr virus G protein-coupled receptor BILF1 is dependent on constitutive signaling

Epstein-Barr virus (EBV) open reading frame BILF1 encodes a seven trans-membrane (TM) G protein-coupled receptor that signals with high constitutive activity through G alpha(i) (Beisser et al., 2005; Paulsen et al., 2005). In this paper, the transforming potential of BILF1 is investigated in vitro in a foci formation assay using retrovirally transduced NIH3T3 cells, as well as in vivo by using nude mice. BILF1 revealed a substantial transforming potential that was dependent on constitutive signaling, as a signaling-deficient mutant completely lost its ability to transform cells in vitro, and an intermediate-active triple-mutated receptor possessed an intermediate transforming potential. Furthermore, BILF1 expression induced vascular endothelial growth factor secretion in a constitutively active manner. In nude mice, BILF1 expression induced vascular endothelial growth factor secretion in a constitutively active manner. In vivo, BILF1 expression induced vascular endothelial growth factor secretion in a constitutively active manner. In vivo, BILF1 expression induced vascular endothelial growth factor secretion in a constitutively active manner. In vivo, BILF1 expression induced vascular endothelial growth factor secretion in a constitutively active manner.
Generating substrate bound functional chemokine gradients in vitro

Microcontact printing (mCP) is employed to generate discontinuous microscale gradients of active fractalkine, a chemokine expressed by endothelial cells near sites of inflammation where it is believed to form concentration gradients descending away from the inflamed area. In vivo, fractalkine is a transmembrane molecule extending its chemokine domain into the vascular lumen. Substrate bound in vitro gradients may thus closely resemble in vivo conditions. Direct mCP of sensitive proteins like fractalkine may cause partial protein denaturation and will not ensure correct orientation of the biologically active part of the molecules. Here, indirect mCP of a capture antibody recognizing a molecular tag on the target protein is successfully used to pattern tagged fractalkine in microscale gradient patterns. Fractalkine functions as an adhesion molecule for leukocytes. Cells expressing the fractalkine receptor are found to attach to the gradient structure at a density correlated with the fractional area covered by fractalkine. This indicates that the patterned fractalkine maintains its biological function. The method can be applied to in vitro studies of cell responses to the wide range of naturally surface-bound chemokines (haptotactic gradients). The use of a capture antibody facilitates control of the orientation of tagged molecules, thereby ensuring a high degree of bio-functionality through correct presentation and reduced protein denaturation.

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Projects:

**Virus fishing in a polymeric microdevice**
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