Oncomodulatory properties of the human cytomegalovirus-encoded receptors US28 and UL33

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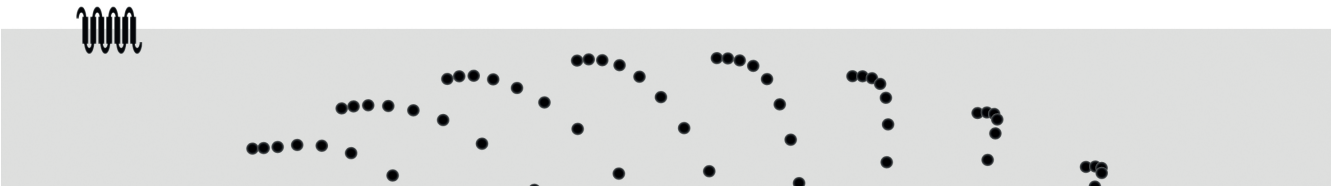
chapter 1

Introduction



Adapted from Slinger, Langemeijer *et al.*
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1.1 The GPCR family

G protein coupled receptors (GPCRs) are the largest family of transmembrane receptors that process extracellular signals into intracellular responses. Recently, Robert Lefkowitz and Brian Kobilka were awarded the 2012 Nobel Prize in Chemistry for their groundbreaking discoveries on this important family of GPCRs [322]. In 1968, Lefkowitz started to use radioactively labelled ligands in order to trace cells' receptors. He managed to unveil several receptors, among those a receptor for adrenalin: the β-adrenergic receptor. During the 1980s Kobilka isolated the gene that encodes the human β-adrenergic receptor. Furthermore, they discovered that the receptor was similar to one in the eye that captures light and realized that there is a whole family of receptors that may function in a comparable manner. Today this family is referred to as G protein-coupled receptors. With the completion of the human genome sequence in 2000, over 800 putative GPCR-encoding genes were identified [8]. Binding- and functional studies of GPCRs have shown the chemically diversity of their ligands [9]. These ligands include amines, peptides, proteins, cell surface adhesion molecules, odorants and photons [10]. Since GPCRs control physiological processes including vision, smell and key biological functions like cellular motility, growth, differentiation and gene transcription, misregulation can lead to pathology [11]. Due to their diverse functionality and prevalent expression in the plasma membrane, GPCRs are important drug targets for the pharmaceutical industry. Notably, more than 30% of all marketed therapeutics target a GPCR [12]. Surprisingly, only a relative small portion of the GPCR family ($\sim$150 members, mainly biogenic amine receptors) is targeted so far. This implies

an enormous potential to exploit the remaining family members, including >100 orphan receptors [13].

GPCR classification and structure

The core protein structure of GPCRs consist of seven transmembrane spanning regions (7TM), hence their alternative name; 7TM receptors. The transmembrane α-helices are connected by three intracellular and three extracellular loops, and flanked by an extracellular amino-terminus and an intracellular carboxyl terminus [14, 15] (Figure 1.1). GPCRs are classified into five main families (A-E): A) rhodopsin, B) secretin, C) glutamate, D) adhesion and E) frizzled/taste2 [8]. The rhodopsin family is the largest and includes the chemokine receptors. These groups include orphan GPCRs, receptors for which the ligand and the (patho-) physiological function remain unknown [16].

The last decade, crystal structures of rhodopsin, ligand-activated GPCRs like the human β2 adrenergic receptor (β_2AR), the A_{2A} adenosine receptor (A_{2A}AR), the chemokine receptor CXCR4 [17], the dopamine D3 receptor [18] and the Histamine H1 receptor [19], as well as the structures of opsin and an active form of rhodopsin were published [20]. This list is growing rapidly, with the recent additions of the S1P1 receptor [21], the δ, κ, μ-opioid receptors and nociceptin/orphanin FQ receptor [22-25]. These high-resolution insights into receptor structures will enable the application of structure-based approaches to develop better drugs.

A variety of protein-modification and engineering approaches have contributed to the advances in GPCR crystallography. Thermostability of the receptor proteins always has been the bottleneck in these studies. Methods of (TM5/TM6) protein stabilization include binding to Fab or incorporation of T4-Lysosyme (β_2AR [26-28], and/or mutagenesis increasing thermostability (A_{2A}AR, [29] and CXCR4 [17]). Other modifications enabling crystallographic studies of GPCRs include truncation of flexible C terminus or ECL3, removal of potential post-translational modification sites and the addition of tags to the receptor.

The structure of the CXCR4 with a bound small molecular compound and comparison to other GPCR structures is depicted in Figure 1.1. As further details of the 3D structure of GPCRs with bound ligands are resolved, efforts targeting specific functional states of GPCRs will emerge. Therefore, new opportunities for drug discovery will emerge as these receptors play prominent roles in pathology.

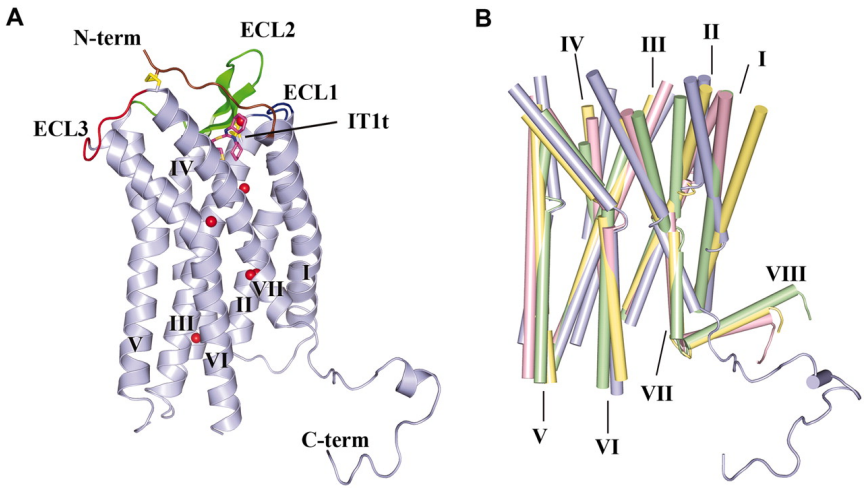

Figure 1.1: Structure model of the CXCR4-small molecule antagonist IT1t-complex and comparison with other GPCR structural models. (A) Structural model of the CXCR4-small compound complex. The receptor is colored blue. The N-terminus, ECL1, ECL2, and ECL3 are highlighted in brown, blue, green, and red, respectively. The small compound is shown in a magenta stick representation. The disulfide bonds are yellow. Conserved water molecules are shown as red spheres. **(B)** Comparison of TM helices for CXCR4 (blue), β_2AR (yellow), A_{2A}AR (green) and rhodopsin (pink). Figure adapted from [17].

GPCR signaling

GPCRs owe their name to their interaction with the heterotrimeric G proteins, which are composed of an α and βγ-subunit. The vast majority of GPCRs are able to transfer signals into cells via G protein coupling. The Gα subunits which define these G proteins are divided into $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$ family members [30]. Classical GPCR-mediated signal transduction involves activation of a heterotrimeric G protein. Upon activation, the Gα protein exchanges bound GDP for GTP. Dissociation and rearrangement of the Gα and βγ subunits further mediates the intracellular signaling [31]. Stimulation (or inhibition) of effector molecules like adenylyl cyclase (AC), phospholipases A2 (PLA2) and C (PLC), and phosphoinositide-3 kinase (PI_3K) results in the production of second messengers that mediate intracellular responses upon receptor activation.

To describe receptor activation in general, a ternary complex model was postulated in 1980 [32]. This model states that GPCR receptor activation requires

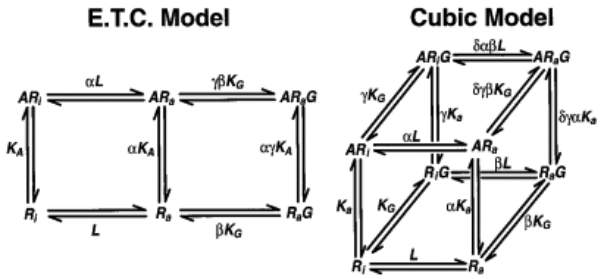

Figure 1.2: Two models of GPCR activation. (A) extended ternary complex model describing two receptor states (R_i and R_a) whereby the active state R_a interacts with G protein (G) [33]. **(B)** the cubic ternary complex whereby both inactive and active states of the receptor are allowed to interact with the G protein, but only AR_aG mediates response [35]. Figure from reference [36].

3 components; an agonist, receptor and a G protein [33]. In their extended, or allosteric ternary complex model, an isomerization step of the inactive receptor (R_i) to an active state receptor (R_a) is required for G protein coupling (Figure 1.2A) [33]. Upon binding of an agonist the equilibrium shifts to R_a. This causes a G protein to bind and leads to downstream signaling. Constitutive activity is defined as the ability of a receptor to undergo agonist-independent isomerization from the inactive (R_i) to the active (R_a) state. This leads to the spontaneous formation of the ternary complex, leading to ligand-independent signaling. Inverse agonists on the other hand, cause a shift of the equilibrium from R_a to R_i, resulting in abrogation of constitutive signaling [34]. Antagonists with equal affinity for R_i and R_a, have no effect on the equilibrium. These neutral antagonists solely block the interactions of agonists or inverse agonists with the receptor. Most likely neutral antagonists are either very weak partial agonists or inverse agonists. Signal transduction via a GPCR is thus determined by cumulative affects of diverse ligands all exerting subtle effects on receptor activity. In 1996 the cubic ternary complex was proposed, in which the G proteins were also allowed to bind to the R_i state (Figure 1.2B) as well, but only AR_aG mediates responses [35].

Besides activation of GPCRs, ligand binding also initiates a series of events to attenuate receptor signaling via desensitization, sequestration and/or internalization. The desensitization process of GPCRs occurs via receptor phosphorylation by G protein receptor kinases (GRKs) [37] and subsequent binding of β-arrestins [38]. The cytosolic β-arrestin proteins translocate to the membrane upon GPCR activation. The subsequent binding of phosphorylated receptors facilitates receptor

internalization via clathrin coated pits [38, 39]. This blocks further G protein-mediated signaling, targets the receptor for internalization or recycling, and redirects signaling to alternative G protein-independent pathways [40]. Additionally to G protein dependent signaling, β-arrestins seem to signal beyond the receptor-arrestin complex, regulating processes like protein translation and gene transcription [41]. Limited information is available on the physiological roles of β-arrestin-dependent signaling. The dual role of β-arrestin makes it difficult to dissociate the consequences of β-arrestin-dependent desensitization from those that might be ascribed to β-arrestin-mediated signaling. Over the past decade, it has become clear that GPCR ligands exist that preferentially stimulate one signal transduction pathway over another. This phenomenon is referred to as functional selectivity or biased signaling [42]. Biased signaling implies that an agonist for one pathway may act as an antagonist or inverse agonist for another signal transduction cascade. Recent evidence generated using β-arrestin knockouts, G protein-uncoupled receptor mutants, and arrestin pathway-selective "biased agonists" is beginning to reveal that β-arrestin signaling plays important roles in the retina, central nervous system, cardiovascular system, bone remodeling, immune system and cancer [41, 43]. Functional selectivity of compounds has been indicated as potentially highly interesting in reducing drug-induced side-effects [43].

Constitutive signaling and pathology

A receptor which is capable of producing its biological response in the absence of a bound ligand is said to display "constitutive activity". However, most GPCRs, including the majority of the chemokine receptors, do not exhibit constitutive activity. Nonetheless, over 60 wild-type (WT) GPCRs, as well as some naturally occurring and disease-causing mutant GPCRs displaying constitutive activity have been identified [44]. For example, mutations in rhodopsin are associated with congenital night-blindness [45], in parathyroid hormone, parathyroid hormone-related peptide (PTH-PTHrP) receptor with short-limb dwarfism or Jansen-type metaphyseal chondrodysplasia [46].

Since GPCRs are able to regulate proliferative signaling, it is surprising that only few are currently associated with cancer development. Examples include some of the muscarinic acetylcholine receptors [47] and the α_{1B}-adrenoceptor [48]. GPCRs are susceptible to constitutive activation by spontaneous somatic mutations and may thus behave as oncogenes. Mutations in IL3 of Thyrotropin receptor (TSHR) for e.g. have been linked to hyperfunctioning thyroid adenomas [49].

GPCR oligomerization

The idea that GPCRs might form dimers or higher order oligomers was first proposed in 1982 [50]. Although most GPCRs are fully capable to induce intracellular signaling upon agonist binding on their own, many appear to exist and function in homo- and/or heteromeric form for at least some time. Besides membrane trafficking from the endoplasmatic reticulum (ER), signaling can also be differently regulated in oligomers. The first proof of GPCRs functioning as dimers, was obtained with the $GABA_B$ receptor, involved in epilepsy, pain, drug addiction, anxiety and depression [51]. Heterodimerization of $GABA_{B_1}$ and $GABA_{B_2}$ is required for the formation of functional receptors, with the $GABA_{B_1}$ providing the agonist binding site and the $GABA_{B_2}$ helping to traffic the $GABA_{B_1}$ to the cell surface and thereby increasing agonist affinity and G protein activation [52]. This crosstalk between GPCRs, also known as negative or positive cooperativity [17], involves binding of a ligand to one protomer, inducing a structural change in the second protomer, which modifies the ligand-binding affinity of this second receptor. Recently, more indications that not one, but two GPCRs are required to bind to one G protein have been presented [53], as well as GPCRs dimer binding to one scaffold protein. Together these data point at a functional unit of two GPCRs, one involved in signal transduction and one additional regulatory receptor.

Chemokine receptors and chemokines

An important subclass of the family A GPCRs is formed by the chemokine receptors. These receptors were initially identified on the surface of cells involved in the immune system as proteins that bind chemotactic cytokines and subsequently initiate chemotactic responses [54]. Chemokine receptors are expressed on a wide range of leukocytes. They play a key role in the regulation of the immune system during homeostatis and inflammation by coordinating migration, activation, degranulation and differentiation of leukocytes [55]. Additionally, chemokine receptors are also present on cells that are of a non-hematopoietic origin such as endothelial cells and neurons [56], smooth muscle cells, epithelial and stromal cells. Furthermore, several chemokine receptors have been reported to be associated with several tumors [57]. This is not unexpected considering the observed angiomodulatory properties of some of the (CXC) chemokine receptor ligands. Their role in organogenesis, angiogenesis and the central nervous system, as well as tumor growth and metastasis, has becomes apparent [58]. For example, CXCR4

has been correlated to more than 23 types of cancers, promoting metastasis, angiogenesis, and tumor cell growth or survival [59]. In addition, chemokine receptors have been identified as co-receptors for cellular entry of primate lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) [60, 61]. The discovery of chemokine receptors CCR5 and CXCR4 acting as co-receptors for HIV-entry [62], boosted chemokine receptor research. Besides these chemokine receptors, also other chemokine receptors as well as e.g. the receptor for N-formylpeptides FPRL1, the orphan GPR1 and the virus-encoded US28 have the potential to act as HIV-1 co-receptors in vivo [63].

The mammalian chemokine (the name is derived from chemotactic cytokines) signaling system is composed of small protein ligands (the chemokines with a size around 8–10 kDa) that bind and activate chemokine receptors. Together, chemokines and chemokine receptors are important mediators of the mammalian immune system. Chemokine nomenclature is based on conserved cysteine motifs and the chemokine family can be subdivided into four families, CCL, CXCL, XCL and CX3CL. In this system, the chemokines themselves are noted with an L at the end and the chemokine receptors with an R at the end. There is a large amount of promiscuity in the chemokine / chemokine receptor system as some chemokines activate multiple chemokine receptors (Figure 1.3). The superfamily of chemokines is currently composed of at least 46 members, of which the majority belongs to either the CCL or the CXCL family. The XCL and CX3CL families only have two and one member, respectively [64].

The main function of chemokines is to attract cells, which express distinct subsets of chemokine receptors. The CCL chemokines attract a variety of cells of the immune system, whereas the CXC chemokines mainly attract neutrophils and lymphocytes [55, 65]. Chemokine receptors primarily activate $G\alpha_i$ proteins, which play an important role in chemotaxis [66]. Additionally, some chemokine receptors activate MAPK and Akt signaling pathways, implicated in proliferation and survival [67]. Furthermore, the CXC chemokines possess angiomodulatory activity [68]. The combination of these angiomodulatory properties and their homing response renders the CXC chemokines to be of special interest in tumor growth as well as in metastasis [69]. Notably, in breast cancer, the combined effects of, e.g., CXCR4 and CXCL12 are of importance for tumor development [70].

Chemokines can be classified based on their inducible (inflammatory) or constitutive (homeostatic) expression. The inducible chemokines are expressed upon injury, infection or inflammatory stimulus. The constitutive chemokines are expressed in the absence of inflammatory stimuli and control cell trafficking in the developing embryo as well as leukocyte homing in immune surveillance. The chemokine system assures that cell trafficking during inflammatory responses oc-

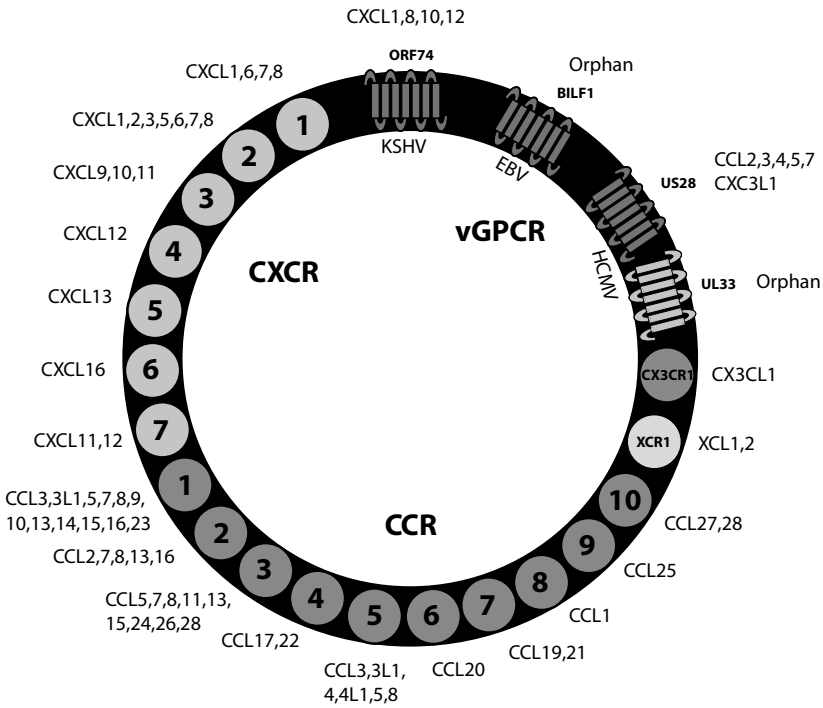

Figure 1.3: Scheme of the chemokine/chemokine receptor system. The different CXC-receptors (1–-7, top left), CC-receptors (1–-10, lower half), XC-receptor and CX3C-receptor (right) with their respective ligands are depicted. Additionally, the viral receptors ORF74 (from KSHV), BILF1 (from EBV) and US28 and UL33 (from HCMV) that are discussed in this thesis are shown with their ligands if known (top right). Figure adapted from [7].

curs in the proper spatial and temporal fashion. Disturbance of this system will lead to inflammatory disease [65]. In some cases chemokine (-receptor) redundancy has been observed. This includes the crossing of class-boundaries, like e.g. the CXCR3 ligands CXCL9, 10 and 11, which are also capable to antagonize CCR3 [71].

1.2 Viral GPCRs

It is clear that chemokine receptors play key roles in pathologies ranging from acting as HIV entry receptors to cancer metastasis. Besides these chemokine receptors, viruses that are able to infect cells are known to encode for receptors that show homology to chemokine receptors. The research described in this thesis involves two virus-encoded GPCRs (vGPCRs); US28 and UL33. Besides these viral receptors, encoded by the human cytomegalovirus, many other herpesviruses contain DNA encoding for GPCRs. In the sections below the different viruses and their GPCRs will be discussed and Table 1.1 gives an overview of virus-encoded GPCRs with their human homologue, putative functions and references.

Herpesviruses and their GPCRs

Mammalian viruses have evolved multiple strategies to evade or subvert the host's immune system. Members of the Herpesviridae family are particularly successful in this respect, with some of them capable of achieving life-long infections in up to 90% of the general population. To be able to do this, several host genes have been 'hijacked' in the course of evolution by these viruses. This 'hijacked' genetic material includes genes encoding chemokines and chemokine receptors. The receptors and chemokines thus obtained have been severely modified and optimized for the virus' own benefit and often exhibit unique characteristics, such as receptors that show promiscuous binding of ligands and constitutive activity. In several cases these vGPCRs have been shown to possess oncogenic properties. Besides the Herpesviridae, the Capripoxviridae family of viruses also possesses vGPCRs [94]. However, not much is known about these proteins, their function-

Table 1.1: Selection of virus-encoded GPCRs with their human chemokine receptor homologs, putative functions and references. CKR stands for chemokine receptor.

Virus	vGPCR	CKR homolog	Putative functions	Ref
HHV4 (EBV)	BILF1	CXCR4	Immune evasion; MHC class I downregulation and PKC signaling. Dispensable for formation of lymphoproliferative diseases.	[97]
			In vitro transforming potential	[98]
HHV5 (HCMV)	US28	CCR1, CCR5 and CX3CR1	HIV infection; mediates virus entry	[99]
			Atherosclerosis; mediates SMC migration via $G\alpha_{12/13}$. FAK and ERK1/2 activation via $G\alpha_{12}$, inducing actin cytoskeleton rearrangement. Initiatation of pro-migratory signaling in SMC via activation of Pyk2.	[100] [101] [102]
			In many cell lines prolonged US28 expression results in apoptosis	[103]
			Immune evasion; sequestering chemokines, IL-6, COX-2 secretion; in vitro transforming potential	[104] [105] [106]
			Constitutive activation of PLCβ and NF-κB, NFAT and CREB via $G\alpha_q$ and $G\alpha_i$, SRE via $G\alpha_q$. Involvement of GASP-1 in US28 signal transduction.	[107] [108] [109] [110]
			Regulation of the viral genome via CREB by stimulating the immediate-early (IE) gene promoter	[111]
			Promotion of intestinal neoplasia via activation of canonical Wnt/β-catenin pathway in trangenic mouse model.	[112]
	UL33	CCR10	Viral life cycle; Murine and rat orthologs important in viral dissemination to and replication in the salivary glands.	[113]
			Able to silence US28-mediated activation of NF-κB	[114]
	UL78	CXCR1	Dispensible for viral replication; Able to silence US28-mediated activation of NF-κB	[115] [114]
	US27	CXCR3	Required for efficient spread by the extracellular route but not for direct cell-to-cell spread	[116] [114]
HHV8 (KSHV)	ORF74	CXCR2	Oncogenesis; 1. Induction of pro-angiogenic and inflamed phenotype; release of cytokines, growth factors and upregulation of adhesion molecules, 2. Transformation of cells, directly or paracrine, 3. Tumor formation in xenograft models and transgenic animals	[117] [81]

ality, and the role they may play in the viral life cycle, so they will not be discussed in this introduction. Although HHV6 and 7 also express vGPCRs, the focus here will be on Kaposi's sarcoma-associated herpesvirus (HHV8), Epstein-Barr virus (HHV4), and cytomegalovirus HCMV (HHV5), which are all capable of setting up persistent latent infections in humans. Two of the herpesviruses, KSHV and EBV, are associated with cancer, while HCMV possesses oncomodulatory potential. The vGPCRs may contribute to the escape of immune surveillance and (constitutively) activate signaling pathways linked to proliferation and inflammation. Some vGPCRs induce activation of autocrine and paracrine signaling, resulting in secretion of growth factors and/or cytokines. As a result, vGPCRs effectively rewire cellular signaling networks. The current state of research on the vGPCRs encoded by these three herpesviruses and their potential involvement in cancer or other associated pathologies will be discussed here. Delineating the cellular

signaling networks modulated by these vGPCRs will be crucial for treatment of virus-associated pathologies.

Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV or HHV8 is a γ-herpesvirus that was first identified in AIDS patients in the 1980s, when frequent occurrences of the usually rare Kaposi's sarcoma (KS) were reported [95]. The virus is endemic in Africa, with infection rates of over 50% in Central Africa, whilst infection rates in the rest of the world are much lower (between 0.2% and 10%). There are currently three different recognized variants of KS. Classic KS occurs mainly in Mediterranean men over the age of 50, and the lesions do not typically spread beyond the extremities. Endemic KS is common in particular parts of Africa. In Uganda, for example, KS accounts for up to 9% of the cancers. Finally, there is AIDS-associated KS, which was found initially predominantly in AIDS-affected patients, a particularly aggressive and often fatal variant of KS. In all the variants, KS is characterized by highly vascularized lesions [96]. Moreover, KHSV is found in nearly 100% of tumor isolates from patients. KSHV is also associated with primary effusion lymphoma and multicentric Castleman's disease [97, 98].

The KHSV genome consists of a double stranded DNA genome, encoding 84 open-reading frames [99] and 12 microRNAs (miRNAs) [100]. Open-reading frame 74 encodes a vGPCR, also referred to as ORF74. ORF74 is a close homologue of CXCR2 and binds many chemokines, particularly those of the CXC family, which is indicative for a role in the avoidance of the host immune system by KSHV. In addition, ORF74 displays constitutive activity, which has been correlated with oncogenesis in vitro and in vivo [101, 102]. Furthermore, transgenic mice expressing ORF74 develop KS-like lesions [5, 93]. Host cell signal transduction pathways that are activated by ORF74 include mitogen-activated protein kinases (MAPKs), phospholipase C (PLC), phosphoinositide-3-kinase (PI3K), and Akt [103]. ORF74 has also been shown to activate NF-κB via PI3K and Akt. Moreover, the activation of NF-κB by ORF74 is accompanied by the release of inflammatory cytokines [104]. The chemokines CXCL8 and CXCL1 act as (partial) agonists upon ORF74 [105]. In contrast, CXCL10 and CXCL12 act as inverse agonists [106-108]. Furthermore, ORF74 induces IL-6 release in infected cells, in the aforementioned manner via NF-κB. Subsequently, IL-6 triggers VEGF production in both a paracrine as well as autocrine manner [104]. Moreover, KSHV encodes a viral IL-6 homologue (vIL-6) that may further strengthen the inflammatory responses in

the infected cells [109, 110]. The increased IL-6 levels induced by ORF74 produce an inflammatory environment that has been shown to promote transformation of cells and formation of tumors [111]. IL-6 secretion and subsequent para- or autocrine activation of the JAK/STAT axis are thought to play an important role in these processes [112, 113]. Interestingly, stimulation of cells neighboring the IL-6 producing cells may also induce an epigenetic switch, a process where a transient event results in a permanent change in phenotype of the affected cells, which has recently been described in a breast cancer model [114]. Recently it was shown that a tumor virus may use a viral protein to interfere with microRNA (miRNA)-mediated repression of a miRNA target to induce cell proliferation and tumorigenesis during virus infection [115]. KSHV lytic infection increases the expression of both viral and human interleukin-6 (vIL-6 and hIL-6, respectively). vIL-6 was demonstrated to be a target of ORF57 and disrupting the ORF57 gene in the KSHV genome leads to inefficient expression due to loss of stabilization of vIL-6 and hIL-6 RNA's [115]. KSHV induced angiogenin controls angiogenesis [116]. It is suggested that LANA-1 (ORF73) and vGPCR ORF74 play roles in KSHV induced angiogenesis. The angiogenic potential of the vGPCR might be also due to its ability to induce angiogenin.

Only a small amount of the tumor cells in KS is actually KSHV positive in early KS. In later stage disease, more than 90% of the tumor cells are KSHV positive [98]. In the latter case, only a small subset actually displays a gene expression profile typical for the lytic phase, including ORF74 expression [117]. Although ORF74 is not the only oncogenic determinant in KSHV, this observation is striking. A small number of ORF74-expressing cells can alter the behavior of the surrounding tumor cells via paracrine signaling by inducing the production of factors like IL-6. Such a feed-forward mechanism creates an inflammatory environment which may be an important factor in KSHV related cancers.

Epstein–Barr virus (EBV)

EBV or HHV4 is another lymphotropic virus involved in proliferative diseases. This γ-herpesvirus was first identified in the 1960s. It infects over 90% of humans and persists during lifetime. Infection with EBV usually occurs by contact with oral secretions, causing infectious mononucleosis, also known as kissing disease. The virus replicates in cells in the oropharynx. B cells in the oropharynx are the primary site of infection and resting memory B cells are thought to be the site of persistence of EBV within the body. Shedding of EBV from the oropharynx is

abolished in patients treated with acyclovir, whereas the number of EBV-infected B cells in the circulation remains the same as before treatment [118].

EBV-related diseases are B cell- and epithelial cell-specific diseases, namely Burkitt lymphoma, gastric carcinoma and nasopharyngeal carcinoma [119]. These diseases are mainly manifested in seemingly immuno-competent individuals. On the other hand, post transplant lymphoproliferative disorders (PTLD), which pose a life-threatening problem in the Western world particularly, are common in immune-suppressed patients like HIV-infected patients and recipients of organ or bone marrow transplants [261]. These immune-compromised patients have impaired T-cell immunity and are unable to control the proliferation of EBV-infected B cells. Additionally, decreased cytotoxic T cell surveillance can lead to an increased risk of developing EBV-associated diseases such as hairy leukoplakia and Hodgkin lymphoma [261].

The EBV genome consists of a linear DNA molecule that encodes approximately 94 viral proteins. During viral replication, these proteins are important for regulation of the expression of viral genes, duplicating viral DNA, forming structural components of the virion, and modulating the host immune response. The EBV proteins expressed during latency differ from those present in cells in which productive EBV-infection and replication takes place. Only a small subset of the viral genes that are active during replication, is expressed in latently infected B cells *in vitro*. Only two types of non-translated RNA, six nuclear proteins, and two membrane proteins are expressed in latently infected B cells [120]. Recently, miRNAs secreted by EBV-infected cells were shown to be transferred to and act in uninfected recipient cells. This resulted in a dose-dependent repression of EBV target gene expression, including that of CXCL11/ITAC, an immunoregulatory gene down-regulated in primary EBV-associated lymphomas [121]. In a co-culture system and in peripheral blood mononuclear cells from patients with increased EBV load, EBV BART miRNAs are present in both B-cell and non-B-cell fractions, suggesting the occurrence of miRNA transfer. Thus, miRNA-mediated gene silencing as a potential mechanism of interfering in intercellular communication between cells of the immune system is exploited by the human γ-herpesvirus EBV [121].

Like in KSHV, the EBV genome also contains a single viral GPCR, BILF1, a gene specifically expressed in the lytic phase of the viral replication cycle. BILF1 displays -low level of- homology to chemokine receptor CXCR4, a chemokine receptor known to be involved in onset and development of different cancers [70]. Attempts that were undertaken to deorphanize BILF1 so far have not been successful. However, downstream signaling by BILF1 can be studied, as this receptor like other vGPCRs, signals in a constitutive manner through $G\alpha_i$ [122, 123]. It has been

suggested that EBV may use BILF1 to control $G\alpha_i$-activated pathways during viral lytic replication, thereby affecting disease progression [122]. Recently, the in vitro transforming potential of BILF1 was shown using retrovirus-transduced NIH-3T3 cells in a xenograft model system. The transforming capacity of BILF1 appeared to be largely dependent on its constitutive signaling [73], like we have previously shown for the HCMV encoded receptor US28. Furthermore, BILF1 expression also induced VEGF secretion in a constitutive manner. These data suggest that BILF1, when expressed during EBV infection, could indeed mediate the pathogenesis of EBV-associated diseases and malignancies. Furthermore, the correlation between constitutive receptor activity and the ability to mediate transformation of the host cell supports the idea that inverse agonists for BILF1 might be relevant therapeutic candidates [73].

The function of BILF1 also has been examined in the context of immune evasion. Expression of BILF1 constitutively inhibits the phosphorylation of RNA-dependent protein kinase (PKR) [123]. Upon viral infection, PKR is activated by phosphorylation, causing the overall cellular translational machinery to stop, thereby prohibiting viral replication [124]. This mechanism serves to prevent viral spreading by elimination of the infected cells. Thus, the inhibition of PKR by BILF1 may facilitate EBV infection preventing a cellular antiviral response. Moreover, it was recently shown that BILF1 reduces the levels of MHC class I at the cell surface of epithelial and melanoma cells [72]. Targeting these molecules for lysosomal degradation, results in impaired recognition by immune T cells. Together with two other EBV genes BGLF5 and BNLF2A expressed in the lytic cycle, BILF1 is the third gene in a group that cooperates to interfere with MHC class I antigen processing. This underscores the importance of the need for EBV to be able to evade CD8+ T cell responses during the lytic replication cycle. The effect on MHC class I degradation is independent of constitutive BILF1 signaling and its molecular mechanisms are distinct. It involves physical association of BILF1 with MHC class I molecules, an increased turnover from the cell surface, and enhanced degradation via lysosomal proteases [72]. Further characterization of the molecular mechanisms by which the BILF1 protein affects its immunomodulatory functions involving the MHC-1 molecules revealed an unexpected complexity. BILF1 also modulates antigen presentation via an additional mechanism which involves diversion of newly synthesized MHC-I molecules en route to the cell surface. These separate effects of BILF1 on both the exocytic and endocytic pathways of MHC-I trafficking causes marked impairment of CD8+ T cell recognition [125].

In addition to the above-mentioned immune-escape mechanisms, viral GPCRs may also affect the properties of human receptors by means of receptor oligomerization and/or downstream signaling cross-talk. GPCR proteins can physically in-

teract with each other, thereby modifying intracellular signaling and cellular functions [126]. B lymphocyte migration and functioning is regulated by chemokines acting on their cognate receptors. We have recently shown using BRET, trFRET and co-immunoprecipitation techniques that BILF1 heterodimerizes with various chemokine receptors, including CXCR4, endogenously expressed in B lymphocytes. The oligomerization of BILF1 with chemokine receptors involved in B lymphocyte migration may change the receptors responsiveness to chemokines, resulting in altered homing and homeostasis of infected B lymphocytes. This might be an essential step for EBV dissemination or in EBV-induced pathogenesis in general [127]. Negative binding cooperativity of both cognate chemokines and small drug-like compounds has been shown for CCR2/CCR5 and CCR2/CXCR4 chemokine receptor heterodimers [128]. Since BILF1 is still an orphan GPCR, such binding cooperativity cannot be investigated at this moment. On the other hand, the active conformation of the constitutive active BILF1 might affect the function of partnering receptors in a hetero-oligomer, as previously shown for other GPCR dimers [53]. By means of its constitutive activity BILF1 might also scavenge $G\alpha_i$ proteins, inhibiting the correct functioning of endogenous (chemokine) receptor in this way. As BILF1 seems to play a role in preventing the immune system from clearing the virus from the body, this may present an extra venue that can be targeted in treatment of EBV infection. Besides viral proteins, viruses also contain miRNA's. For EBV it has been shown that viral miRNAs are delivered to neighbouring cells via exosomes [121]. Which (host) mRNAs are targeted by the viral miRNAs for degradation is currently only partially known. However, it implies there is a whole new mechanism of signal transduction available to the viruses, which could also have impact on the immune system.

Human cytomegalovirus (HCMV)

Another member of the Herpesviridae family and β-herpesvirus subfamily is HCMV (HHV5). HCMV is widely present among the general population, with up to 50-80% of the individuals harboring a latent infection [129]. While HCMV infection is asymptomatic in immune-competent individuals, it can cause severe pathologies in immune-compromised patients [3, 130]. HCMV infection during pregnancy can result in aberrant development of the unborn child resulting in, for example, hearing loss [131], [132]. Furthermore, HCMV infection has been correlated to several pathologies, such as arthrosclerosis [133].

The HCMV genome is the largest of any human virus known so far, being 236

kb in size. The genome can be divided into two parts, a long part and a short part named UL and US, respectively. Also, a number of miRNAs have been found to be present on the HCMV genome [134]. HCMV proteins, DNA and mRNA have been detected in multiple tumors which, together with epidemiological data, suggest a role for HCMV in cancer [135, 136]. Furthermore, HCMV preferably infects cancer cells [137]. However, unlike KSHV and EBV, HCMV is not considered an oncogenic virus and it is unlikely that HCMV by itself can act as an oncogenic factor. In post transplant lymphoproliferative disorders (PTLD) an etiological role for HCMV is unlikely, since PTLD risk and CMV antiviral drugs were found not to be associated in a large multicentre study [138]. Alternatively, HCMV may have an oncomodulatory role in other (virus-associated) cancers, to catalyze oncogenic processes that have already been initiated or to suppress the immune system.

Recently, CMV was confirmed as cause of the most common salivary gland cancers [4, 139]. Thereby, CMV joins a group of fewer than 10 identified oncoviruses, as a virus that can either trigger cancer in healthy cells or exploit mutant cell weaknesses to enhance tumor formation.

Studies in both human salivary gland tumors and salivary glands of postnatal mice show that the CMV in the tumors is active, but also that the amount of virus-created proteins found is positively correlated with the severity of the cancer. How reactivation of the dormant virus in the salivary glands takes place is still unsolved.

After salivary glands obtained from newborn mice were exposed to purified CMV, cancer developed. In addition, efforts to stop the cancer's progression identified how the virus was acting upon the cells to spark the disease. The specific molecular signaling pathway exploited by the virus to create tumors, normally active during embryonic growth and development, appears to be the same in humans and mice. When CMV turns it back on, the resulting growth is a malignant tumor that supports production of more of the virus.

With the new information about CMV's connection to cancer comes hope for new prevention and treatment methods. The mouse salivary gland model created to connect CMV to cancer might also be used to design more effective treatments [140], allowing more rational design of drugs used to treat CMV-induced tumors. Much more information about viruses and their connections to cancer and other health issues seemingly unrelated to viral infection are expected to emerge in the near future.

HCMV vGPCRs and signal transduction

In contrast to KSHV and EBV, the HCMV genome encodes four vGPCRs; US27, US28, UL33, and UL78. All of these genes encode chemokine receptor homologues, indicating that they were most likely pirated from the human- or another

mammalian-genome at some point during evolution [141-143]. Homologues of UL33 and UL78 are conserved throughout the β-herpesvirus family, whereas homologues of US28 and US27 have been identified only in primate CMVs closely related to HCMV. The US28 homolog of rhesus macaque CMV homolog RhUS28.5 has been found in the viral envelope [144]. Both UL33 and US28 were found to co-localize with the viral envelop glycoproteins gB and gH [145, 146]. Although UL33 [145] and US28 [147] are dispensable for viral replication in respectively MCR5 and human forskin fibroblasts, functional consequence of expressing vGPCRs on the viral particle may enhance binding of the virus particles to target cells. Alternatively, these receptors could exert their effect by rewiring cellular signaling of the host cells.

In Chapter 4 we show that infected cells bind CCL5 directly after infection, well before any US28 has been synthesized from the viral genome. Presence of vGPCRs on the viral particle likely enhances binding to target cells [148], considering that US28 recognizes the membrane-bound chemokine CX_3CL1 [149].

Recently, five amino acids of US28 were found to be under strong positive selection to increase immune adaptation properties of the virus [150]. Three of these, E18D/L, D19A/E/G, and R267K/Q, were located in the extracellular domains and play a crucial role in successful viral adaptation in response to the host's immune defense [150]. These domains are located in close proximity of the chemokine binding site between the residues 11 till 16 [151]. Additionally, the existence of polymorphisms among the US28 genes of clinical isolates of HCMV from infants with suspected congenital infection was demonstrated [148].

Analysis of the US28 amino-acid sequence showed that almost all of the important functional groups were highly conserved. However, the mutations found were concentrated at the N (AA8) and C termini (AA 310 and 287) and at the ligand binding site of this viral GPCR (AA15, 18, 19, 21 and 25). Comparison of US28 sequences from AIDS patients with those from children showed that both sequences have their own specific high mutation points [148]. The high mutation sites in US28 from children were mainly located at the C terminus (AA 310 and 287), whereas those from AIDS patients were mainly located at the N terminus (AA8, 15, 21 and 24). Phylogenetic analysis divided the 34 sequences available into three groups, which showed correlation with the observed pathologies (megacolon, jaundice vs AIDS-related pathologies), suggesting that the occurrence of mutations could be associated with pathogenesis. Infection in children occurs mainly in the digestive and nervous systems, whereas in AIDS patients, infection mainly occurs in the lungs and eyes. US28 can bind chemokines and play an important part in the attraction and activation of leukocytes. The migration of leukocytes can transport infected cells to different parts of the body. The intracellular

C terminus of the US28 receptor is involved in endocytosis of the protein [152]. Differences in sequence mutations in clinical isolates from children and AIDS patients could affect the US28-related migration of leukocytes and lead to different tissue and organ targets.

Only US28, and not the HCMV-encoded US27, UL33 and UL78 have been shown to bind to chemokines [79]. US28 binds CCL5 (RANTES) and CCL2 (MCP-1), of the CC chemokine family [153, 154], and also CX_3CL1 (fractalkine) [149]. Depletion of chemokines from the medium was demonstrated after HCMV infection [79]. Using competitive binding studies HCMV-infected cells, and in particular US28-expressing cells were shown to bind chemokines. Cells infected with a US28-deleted HCMV mutant strain failed to remove extracellular accumulating CCL5 and CCL2. The same holds true for HCMV lacking both US27 and US28 genes. Cells infected with HCMV receptor deletion mutant strains (ΔUS27 and/or ΔUL33) continued to bind and downregulate CCL5 and CCL2 and CCL3, respectively. Biotinylated CCL5 was also continuously internalized and accumulated in wild type HCMV-infected cells. Thus, HCMV can modify the chemokine environment of infected cells through sequestering and internalization of CC chemokines, principally by expression of the US28-encoded chemokine receptor [79].

Upon binding of chemokines, US28 is rapidly internalized, reflecting a possible function as a chemokine sink in the viral life cycle [155]. Monocyte chemotaxis was induced by supernatants from cells infected with a HCMV US28 deletion mutant, due to endogenously produced chemokines CCL2 and CCL5. However, in HCMV-infected cells that did express US28, these chemokines were sequestered from the supernatant. [155]. Like the viral receptors ORF74 and BILF1, US28 displays constitutive signaling, activating PLC and NF-κB [82] as well as NFAT and CREB [83] via $G\alpha_q$ and $G\alpha_i$. The constitutive signaling through CREB has been found to play a role in the regulation of the viral genome, specifically by stimulating the immediate-early gene promoter [86]. Figure 1.4 depicts the different ways three virus-encoded chemokine receptors described above take over signaling in their respective target cells.

In this thesis, signal transduction of the viral GPCRs US28 and UL33, both displaying constitutive activity [82, 156], is investigated. Previously, US28 has been described to bind the inflammatory chemokines CCL2, CCL3, CCL4, CCL5, CCL7 and CX3CL1 [79]. Through internalization US28 acts as a chemokine sink, binding and removing the abovementioned chemokines from the cellular environment, thereby interrupting the immune response that is evoked by the host cell upon viral infection [79]. Similar to other chemokine receptors, such as CCR5 and CXCR4, US28 can act as a co-receptor for HIV-1 entry when co-expressed with CD4 [74]. As a common feature of HIV coreceptors, tyrosine residues are present flanked by

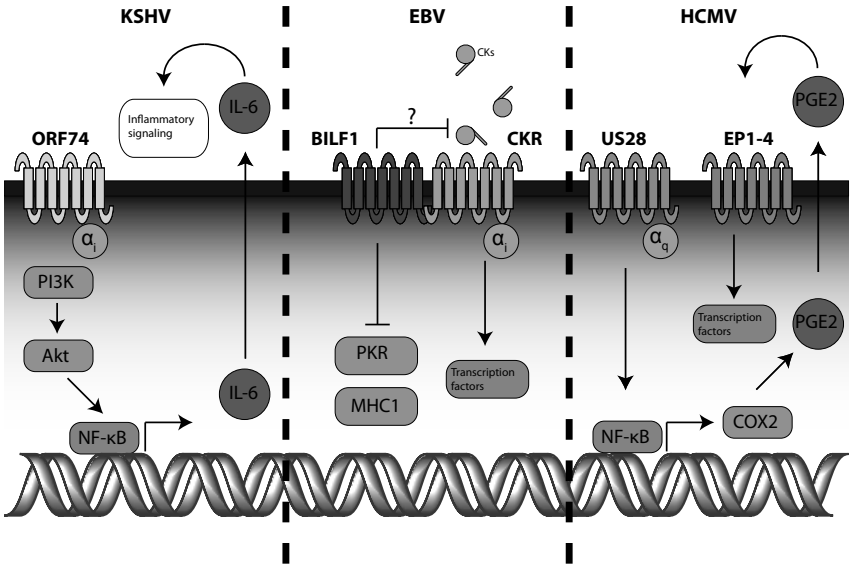

Figure 1.4: The different methods by which the different viruses take over cellular signaling. Showing from the left to the right; ORF74 activating IL-6 transcription in a PI3K and Akt dependent manner. BILF1 displays constitutive $G\alpha_i$ signaling and downregulates PKR and MHC1. When forming dimers with endogenous chemokine receptors it may show negative binding cooperativity. US28 induces NF-κB, which is able to activate COX-2 (Cyclooxygenase-2 or Prostaglandin Synthase 2 (Ptgs2). This can result, via the release of prostaglandin E2 (PGE2), in the activation of VEGF and cyclin D1, proteins involved in angiogenesis and cell cycle regulation respectively (see Chapter 2). In the cases described above, cellular signaling networks have been rewired to the benefit of the virus. In some cases radical alterations of cellular signaling can have unforeseen consequences and may result in or aggravate a transformed phenotype. These viral receptors provide valuable insight into cellular pathways important in virus-associated pathologies and may prove in the future even to be promising drug targets for treatment or prevention of these virus-associated pathologies. Figure from [7].

asparagines (N), aspartic acids (D) or glutamic acids (E) in the amino-terminal extracellular regions. US28 contains Y16 in its ligand binding domain, flanked by EFDYDDE [151].

US28; ligand-dependent signaling

Besides the constitutive signaling, US28 is also able to induce ligand-dependant signaling. Cells transfected with US28 were shown to induce intracellular calcium signaling upon chemokine stimulation by CCL5 (RANTES) and, with lower efficacy, by CCL2 (MCP-1) [154]. Stable expression of US28 in HEK293 cells with and without the heterotrimeric $G\alpha_{16}$ indicated that US28 couples to both $G\alpha_i$ and $G\alpha_{16}$ proteins to activate intracellular calcium flux in response to the chemokines CCL5 and CCL7 (MCP-3) [157]. Notwithstanding several attempts to regenerate these HEK293 stable cell line expressing US28, we were not able to generate these cell lines due to induction of apoptosis [78].

Furthermore, the MAPK extracellular signal-regulated kinase (ERK) is activated in cells that co-express US28 and $G\alpha_{16}$ upon CCL5 stimulation. Thus, through expression of US28, HCMV uses resident G proteins of infected cells to manipulate cellular responses stimulated by several chemokines [157].

A key cellular response mediated by chemokine receptors is induction of chemotaxis. HCMV can infect epithelial cells, fibroblasts, smooth muscle cells, and leukocytes in vivo. Cellular migration induced by US28 was investigated and showed that US28 mediates human vascular smooth muscle cell (SMC) migration in response to either CCL5 or CCL2 via $G\alpha_{12}$-mediated RhoA activation [158, 159]. CX_3CL1 (fractalkine) binding to US28 inhibits the ability of CC-chemokines to induce SMC migration [76]. CCL5, CCL2, and CX_3CL1 binding to US28 induced similar levels of FAK activation in fibroblasts. Overexpression studies indicated that CCL5-mediated stimulation of FAK occurs via a $G\alpha_{12}$-dependent mechanism, while CX_3CL1 utlilizes $G\alpha_q$. In contrast to SMC, when US28 is expressed in macrophages, CX_3CL1 stimulation produces robust migration. CX_3CL1 induces migration of macrophages [76], while CCL5 inhibits migration. Thus, US28-signaling is ligand-specific and cell type-specific. CCL5 and CX_3CL1 promote differential G-protein coupling, leading to the activation of alternative signaling pathways depending on the cell type and the complement of endogenously expressed G-proteins.

In addition, cotransfection of $G\alpha_{12}$ potentiates US28-activated focal adhesion kinase (FAK) and ERK1/2 phosphorylation, thereby inducing actin cytoskeleton rearrangement [76]. Recently, it was shown that CCL5-induced US28 also initiates pro-migratory signaling in SMC via activation of Proline-rich Tyrosine Kinase 2 (Pyk2 or PTK2B) [77]. Autophosphorylation of this FAK-related PTK via US28

induced activation of RhoA is involved in US28-mediated cellular motility. The promiscuous G protein coupling displayed by US28 as well as the wide range of chemokines binding this viral receptor hints at a plethora of potential signaling pathways in which US28 may be involved.

US28; ligand-independent signaling

Besides the ligand-mediated activation of signaling pathways, US28 is known to signal in a constitutive manner. Our group has demonstrated the constitutive activation of phospholipase C (PLC) and NF-κB by US28 [82]. These signaling pathways involve $G\alpha_q$ and $G\beta\gamma$ subunits. Because constitutive US28 activation of signaling is not pertussis toxin (PTX) sensitive, $G\alpha_{i/o}$ seems not involved. US28 is also capable to constitutively activate cAMP via CREB (via $G\alpha_s$) as well as NFAT transcription factors (via $G\alpha_q$ and partially via $G\alpha_i$ (PTX sensitive) through MAPK-dependent pathways [83], and SRE activation by constitutive activation via $G\alpha_q$ [84]. N-terminal deletion of the US28, removing the chemokine binding site, still results in PLC activation. comparable to HCMV-infected cells expressing the wild type US28 [160]. An overview of signaling pathways activated by US28 and other vGPCRs is given in Table 2.

Interestingly, US28 has been shown to possess tumorigenic properties when expressed in a NIH-3T3 murine fibroblast cell line, stimulating both cell proliferation and production of the angiogenic growth factor VEGF. In a mouse xenograft model, tumors were formed when US28-expressing NIH-3T3 cells were injected [6]. US28-induced NF-κB activation leads to release of PGE2 and via COX-2 results in the activation of VEGF and cyclin D1. This signaling cascade can be perturbed using pharmacological inhibitors of COX-2 (e.g., Celecoxib) as described in Chapter 2 of this thesis [81]. These data suggest a role for US28 in HCMV-related cancers that may be reminiscent of that of ORF74 in Kaposi's sarcoma, with the important distinction that HCMV, and thus US28, are probably not direct causative agents. However, as stated above, US28 most likely has an oncomodulatory role. This is strongly suggested by the observations that in many cell lines prolonged US28 expression results in apoptosis [78], indicating an interference of US28 in critical signaling pathways which, depending on cellular context results in either proliferation or apoptosis. It remains to be seen whether US28 also induces a local environmental change reminiscent of ORF74, but the US28-induced release of PGE2 suggests such a possibility. The fact that NF-κB is activated by US28 supports this notion, since NF-κB is known to induce the secretion of inflammatory factors. Recently, we showed US28 activates the IL-6-JAK1-STAT3 signaling axis also through activation of NF-κB and the subsequent production of IL-6 [80]. Analyses of tumor specimens from glioblastoma patients demonstrated

co-localization of US28 and phosphorylated STAT3 in a vascular niche in these tumors. Moreover, increased phospho-STAT3 levels correlated with poor patient prognosis. Therefore, it is proposed that US28 induces proliferation in HCMV-infected tumors, establishing a positive feedback loop through activation of the IL-6-STAT3 signaling axis. Recently, US28 was found to promote an invasive and angiogenic phenotype in glioblastoma [161].

Transgenic mice expressing US28 in intestinal cells revealed that US28 promotes intestinal neoplasia [87]. The Wnt/Frizzled, or β-catenin signaling pathway was found to be involved in this process. In Chapter 3 of this thesis an alternative activation mechanism of β-catenin signaling in US28-expressing cells is presented.

US28 is shown to be able to interact with GPCR-interacting proteins like GASP-1 [85]. The GASP family consists of important cellular determinants that not only regulate the post-endocytic trafficking of GPCRs, but are also able to regulate signaling capacities [162]. Also in the case of US28 GASP-1 mediates changes in post-endocytic degradation thereby affecting signaling capacities of the receptor [85]. Disruption of the GASP-1/US28 interaction inhibits the lysosomal targeting of US28 and diminishes the rate of its post-endocytic degradation. Knock-down of endogenous GASP-1 also affects US28-mediated signaling by impairing the US28-mediated $G\alpha_q$/PLC/inositol phosphate (IP) accumulation as well as the activation of the NF-κB and CRE transcription factors. Overexpression of GASP-1 enhances both IP accumulation and transcription factor activity. Besides GASP-1, the link between US28 with β-arrestin and its involvement in the constitutive active signaling [163] as well as for phosphorylation by GRKs might help in understanding US28-mediated signaling.

Although many interesting features have been described for US28, its biological role in the HCMV life cycle is elusive as yet. The gene is not required for viral replication in vitro, thus suggesting that US28 is dispensable for lytic replication [147]. US28 might play a role during latent infection, dissemination and reactivation of the virus. With mutant HCMV strains lacking US28, UL33 or both the vGPCRs being available, the role of these receptors, can be studied more extensively. The receptor-mediated signaling that is induced in infected hosts might give clues to potential intervention points in HCMV-induced transduction signaling. The link between US28 and COX-2 is described in further detail in Chapter 2.

UL33, US27 and UL78 function

Another vGPCR encoded by HCMV is UL33, which has been shown to signal in a constitutive manner via $G\alpha_q$, $G\alpha_i$, and $G\alpha_s$ [156]. UL33's homolog in mice M33 functions in viral dissemination within the host. Interestingly, a ΔM33 MCMV (viral strain lacking expression of M33) strain can be complemented with UL33,

which suggests functional homology. Therefore, UL33 may serve a role in the HCMV life cycle [88]. The presence of UL33 on the HCMV virion [145] supports such a role for UL33 in HCMV, amongst other putative roles (Figure 1.5). However, in experiments in vitro with viral strains lacking either US28 or UL33 (ΔUS28 and ΔUL33, respectively) no reduced infectivity was observed [147]. Of note, in vitro there is no real necessity for viral dissemination and UL33 would not be necessary.

The biochemical properties of the remaining two vGPCRs, US27 and UL78, remain thus far largely uncharacterized. US27 has been shown to localize on the plasma membrane and in endosomes [146]. Recently, US27 was shown to be required for efficient spread by the extracellular route but not for direct cell-to-cell spread [91]. Additionally, receptor chimeras demonstrated that the C-terminal domain of the human cytomegalovirus US27 gene product is necessary and sufficient for intracellular receptor localization [164]. Their results indicate that the C-terminal domain of the HCMV US27 protein, which contains a di-leucine endocytic sorting motif, causes the intracellular localization (compared to CXC_3R C-tail). This may also explain why no cellular ligands have yet been identified for this viral receptor.

The mouse and rat homologues of UL78, M78 and R78 respectively, have been shown to contribute to efficient viral cell–cell spread [165, 166]. Recently, UL78 of the human cytomegalovirus was shown to form oligomers and traffic between the plasma membrane and different intracellular compartments [167]. Also recently, UL33 and UL78 were shown to heteromerize with host CCR5 and CXCR4 chemokine receptors, impairing their HIV coreceptor activity [168]. Therefore, the role of these vGPCRs might also lay in regulation of membrane expression levels of other (chemokine) receptors and thereby influencing their signal transduction properties.

US27, UL33 and UL78 may have an important regulatory capacity on the function of US28 and therefore in the viral life cycle. Recently the possible interaction / heteromerization of US27, UL33 and UL78 with US28 and the functional consequences thereof were investigated. US27, UL33 and UL78 were found not only to co-localize, but also heteromerize with US28 in vitro [89]. While the constitutive activation of the US28-mediated $G\alpha_q$/PLC pathway was not affected by receptor heteromerization, UL33 and UL78 were able to silence US28-mediated activation of the transcription factor NF-κB.

vGPCRs as drug targets

vGPCRs represent a relatively unexplored class of potential drug targets. Molecular interactions between chemokines and the HCMV-encoded US28 receptor were

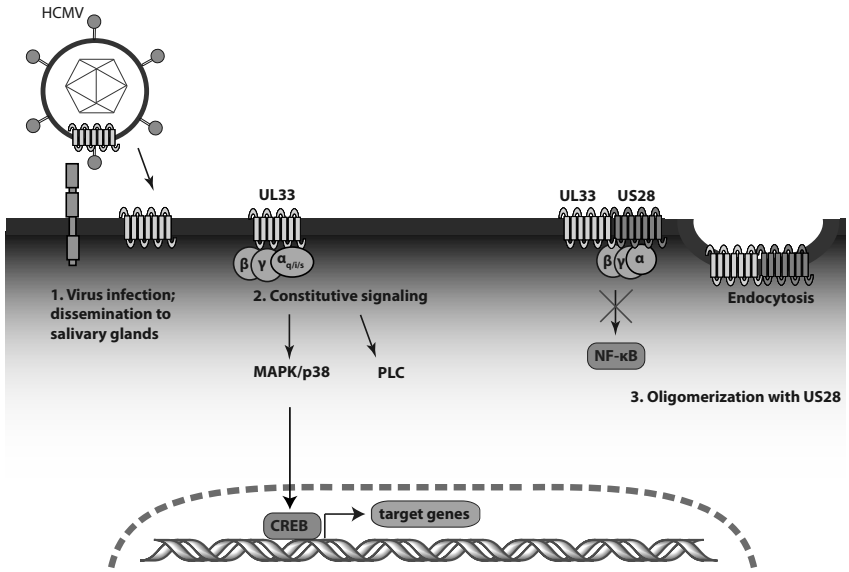

Figure 1.5: Putative roles for the HCMV-encoded receptor UL33. 1. Virus infection by expression on the virion [146]. UL33 is also potentially involved in dissemination to and replication in the salivary glands (in MCMV studies with UL33 replacing the M33) [88]. 2. Constitutive activation of signal transduction pathways (PLC ($G\alpha_q$), CRE ($G\alpha_i$ and $G\alpha_s$) as well as the Rho/p38 pathway via $G\beta\gamma$, [156]). 3. Heteromerization partner for US28, thereby negatively affecting US28-driven NF-κB, but not PLC activation [89].

investigated using a dual approach, consisting of the analysis of both receptor and ligand mutants [151]. In that study several amino acids located in the N terminus of US28 that differentially contribute to the high affinity binding of CC versus CX3C chemokines were identified. Additionally, the importance of secondary modifications occurring at US28, such as sulfation, for ligand recognition was shown. Finally, the effects of chemokine dimerization and interaction with glycosaminoglycans (GAGs) on chemokine binding and activation of US28 were investigated as well using CCL4 as model ligand [151]. Hulshof et al. described the synthesis and pharmacological characterization of the first class of inverse agonists acting on the HCMV-encoded receptor US28 [169, 170]. These inverse agonists served as valuable tools in research on the role of constitutive signaling of US28. In the pathogenesis of HCMV infection they may have therapeutic potential as leads for new antiviral drugs. However, the efficacy of compounds like VUF2274 needs to be

improved to reach nanomolar ranges. Recently, novel nonpeptidergic chemotypes were screened and several inverse agonists and neutral antagonists acting on US28 were identified. These compounds allosterically inhibit chemokine binding and inhibit receptor-mediated PLC signaling [171]. Additionally dihydroisoquinolinone and tetrahydroisoquinoline scaffolds were found to be promising lead structures for novel US28 allosteric inverse agonists [172]. Besides targeting the receptors, the signal transduction pathways activated by vGPCRs can be targeted as well. Activation of the FAK-related Proline-rich Tyrosine kinase 2 (Pyk2) could for instance be targeted, in order to reduce US28 induced pro-migratory signaling, which might be involved in glioblastoma cell migration (Pyk2 deficient cells invade only locally) [77].

Although the three herpesviruses described above all have hijacked chemokine receptors, the role these vGPCRs play in the viral life cycle and the impact they have on host cell's signaling differ widely. Figure 1.4 depicts the different ways three virus-encoded chemokine receptors described above take over signaling in their respective target cells. To summarize, KSHV's ORF74 induces NF-κB signaling via PI3K and its downstream kinase Akt, resulting in IL-6 release and the establishment of an inflammatory environment, beneficial for tumorigenesis. EBV's BILF1 appears to be mainly involved in immune avoidance, which is demonstrated by both the hijacking and subversion of both the PKR and the MHCI signaling pathways. In addition, BILF1 may act as a chemokine receptor scavenger, possibly modulating chemokine receptor affinity for their ligands by means of negative binding cooperativity. This is in contrast with ORF74 and US28, that both negatively influence the immune system by binding the chemokines themselves. The HCMV encoded vGPCR US28 has been shown - at least in a specific cellular context - to be able to induce a proliferative phenotype. US28 may exert this effect by creating a pro-inflammatory microenvironment by activation of COX-2 expression leading to prostaglandin E2 (PGE2) production. Furthermore, by modulating signaling through ROCK via ligand-dependent activation of $G\alpha_{12}$, US28 is also able to promote smooth muscle cell migration. In cells without $G\alpha_{12}$ US28 is not able to induce migration, further underlining the importance of cellular context. Besides US28, HCMV also encodes for the constitutive active UL33, and two other GPCRs. All three are able to heterodimerize with US28. However, the presence of UL33 reduces the US28-induced activation of NF-κB [89].

Proliferative signal transduction induced by US28 and UL33 is further looked into and described in this thesis in **Chapters 2**, **3** and **4**. In **Chapter 5** we describe a preliminary epigenetic analysis of the US28-expressing NIH-3T3 cells and possible consequences hereof for proliferation.

Potential oncogenic signaling pathways activated by vGPCRs

vGPCRs have been shown to constitutively activate signaling pathways linked to proliferation. In this thesis we will examine the ability of US28 and UL33 to activate the COX-2 and β-catenin signaling pathways.

COX-2 signaling

Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX), is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. There are two isozymes of PTGS: a constitutive COX-1 (PTGS1) and an inducible COX-2 (PTGS2), which differ in their regulation of expression and tissue distribution. COX-1, the constitutive form, regulates angiogenesis in endothelial cells and is involved in cell signaling and maintaining tissue homeostasis. COX-1 is normally present in various areas of the body. In the stomach, COX-1 promotes the production of the natural mucus lining, protecting the inner stomach and contributes to reduced acid secretion and pepsin content [173, 174]. Alternative splicing of the COX-1 gene generates two transcript variants. However, the COX-1v or COX-3 does not yield a functional protein in human. COX-2 encodes the inducible isozyme. Its expression is regulated by specific stimulatory events associated with inflammation and mitogenesis. COX-2 is commonly expressed in premalignant lesions, as well as in tumors in colon, lung, head, neck and breast [173], contributing to the pathogenesis of several forms of cancer. In the early 2000's it was proposed as a novel drug target for cancer chemotherapy [174]. COX-2 has been suggested to participate in virally-induced neoplasia related to herpesviruses EBV and KSHV [175]. Interestingly, HCMV-infected cells also show increased levels of COX-2 mRNA [176]. Interestingly, inhibition of COX-2 blocks HCMV replication [177].

Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. The main COX inhibitors are the non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and ibuprofen. These COX inhibitors are not selective and inhibit both types of COX. The resulting inhibition of prostaglandin and thromboxane synthesis reduces inflammation and has antipyretic, antithrombotic and analgesic effects. Selectivity for COX-2 is the main feature of Celecoxib, rofecoxib and other members of this newer NSAID drug class called coxibs. Selective COX-2 inhibitors are associated with a moderately increased risk of vascular events, mainly due to a twofold higher risk of myocardial infarction. Also the high-dose regimens of some traditional NSAIDs (diclofenac and ibuprofen) are associated with a similar increase in risk of vascular events.

As previously mentioned, US28 induces NF-κB, which in its turn is able to

activate COX-2 expression levels. In Chapter 2 the role of this increase of COX-2 is investigated making use of its pharmacological inhibitor Celecoxib. The role of the host-cell genes in HCMV replication and pathogenesis remain highly speculative. The ability to identify genes encoding proteins that play potential mechanistic roles in infectious disease processes underscores the utility of gene array technology in the study of pathogens. DNA array analysis is still an important tool to investigate molecular mechanism involved in pathological processes. Changing patterns of gene expression after HCMV infection were monitored with oligonucleotide arrays [176]. Shenk and co-workers identified upregulation of COX-2 gene expression upon HCMV infection. Using DNA microarray analyses, we identified COX-2 overexpression in US28-expressing cells. This leads to the detailed study of the US28-COX-2 link (Chapter 2). Additionally, overrepresentation of various genes implicated in the β-catenin pathway was found by means of gene array technology using the US28-expressing cells. Therefore, the β-catenin pathway will be discussed in more detail below and the link of US28 to this important tumor-related pathway is described in Chapter 3.

β-catenin signaling

The Wnt/Frizzled or β-catenin signaling pathway was first described in Drosophila for its role in embryogenesis. This pathway is additionally extensively described in many reviews for the role it plays in intestinal cancer [178]. Multiple proteins are involved in the canonical or classical, most studied β-catenin signaling pathway and several non-canonical, less well defined pathways. The central player in the Wnt pathway is the transcriptional regulator β-catenin. Cellular β-catenin levels are tightly regulated. In the absence of pathway activation, a complex of proteins, including the tumor suppressors APC, axin, and the serine kinase glycogen synthase kinase 3β (GSK-3β), phosphorylates β-catenin, marking it for degradation by the proteasome. The extracellular activating ligands for this pathway are a class of molecules referred to as the Wnt factors. Wnt proteins are a major class of secreted ligands of profound importance in establishment of the pattern of development in the bodies of multicellular organisms (embryogenenis). When secreted, Wnt molecules bind to their GPCR receptors on the cell surface called Frizzled (Fz in *Drosophila*, FZD in human) receptors inhibiting the APC–axin–GSK-3β degradation complex. As a result, β-catenin is not targeted for degradation, stabilizes and translocates to the nucleus. After interaction with nuclear transcription factors like TCF-4, the transcription of specific target genes that drive cell proliferation is activated. In the canonical pathway, Dishevelled (Dsh in *Drosophila*, DVL in human) is required to functionally inhibit the activity of the degradation complex and thereby stabilize β-catenin. Besides a key function in transcriptional activation, β-catenin

also fulfills other tasks. Part of the β-catenin proteins in the cell associate with E-cadherin and the actin cytoskeleton at the intracellular site of the membrane, to promote cell adhesion and control cell shape.

The two best-studied non-classical β-catenin activation pathways are the Planar Cell Polarity (WNT/PCP) and Wnt/Calcium (WNT/Ca^{2+}) pathways. PCP refers to coordinated polarization of cells in the plane of a cell sheet. The PCP pathway is involved in rearrangements of the cytoskeleton in order to coordinately move cells to specific locations in the body. In this pathway, Wnt ligand binding to the receptor recruits DVL, which forms a complex with Daam1, activating the small G protein Rho through guanine exchange factor (GEF). Rho activates ROCK (Rho-associated kinase), one of the major regulators of the cytoskeleton. DVL forms a complex with Rac1, which activates JNK and can also lead to actin polymerization. DVL also mediates profilin binding to actin, which can result in restructuring of the cytoskeleton [179].

In the Wnt/Ca^{2+} pathway, Wnt5a and Frizzled receptors regulate intracellular calcium levels [180]. Ligand binding causes activation of PLC, leading to the generation of DAG and IP3. IP3 binding to its receptor on the endoplasmic reticulum increases intracellular calcium concentration. Ligand binding also activates cGMP-specific phosphodiesterase (PDE), depleting cGMP and further increasing the calcium concentration. These increased concentrations of calcium and DAG can activate cell division control protein 42 (Cdc42) through PKC, an important regulator of cell adhesion, migration, and tissue separation. Additionally, increased calcium activates calcineurin and CamKII (calcium/calmodulin-dependent kinase). Calcineurin induces activation of transcription factor NFAT. CamKII activates TAK1 and NLK kinase, which can interfere with TCF/β-catenin signaling in the canonical pathway.

Differences between the canonical and non-canonical pathways include the specific ligands activating each pathway (Wnt4, Wnt5a, and Wntll), β-catenin-LRP5/6 co-receptor involvement and the ability of the non-canonical pathway to inhibit the canonical pathway. Although Frizzled receptors are seven-transmembrane receptors, they remain unconventional [181]. Various aspects of the main Wnt signaling routes remain obscure. For example, it is still unclear whether the FZD/PCP and FZD/Ca^{2+} pathways are distinct or overlapping signaling avenues. Indeed, the involvement of DVL in the FZD/PCP versus FZD/Ca^{2+} pathway, as well as the involvement of heterotrimeric G proteins, are still matters of debate [181].

Activation of β-catenin signaling has been detected in various cancers. Sporadic colorectal cancer i.e. is initiated by the mutation-driven activation of the Wnt pathway, typically the loss of the APC- tumor-suppressor gene. This event

leads to high levels of β-catenin in the cytoplasm and nucleus and to inappropriate activation of target genes like cyclin D1 and cMyc. The association of ulcerative colitis with colorectal cancer is not believed to result from a genetic underlying predisposition, but more likely to be inflammation driven. However, the underlying molecular mechanisms have remained obscure. NSAIDs act through the β-catenin pathway to inhibit the progression of colon cancer by inhibiting the cyclooxygenases and, hence, the production of prostaglandins, upregulated in inflammation. A role for inflammatory prostaglandins, biosynthesized by cyclooxygenases, directly impinging on the Wnt pathway [182] suggested that prostaglandin E2, on binding its receptor, activates a cytoplasmic G protein–coupled receptor ($G\alpha_s$). This in its turn binds axin, a component of the complex that phosphorylates β-catenin. This event may lead to dissociation of the complex, the accumulation of unphosphorylated β-catenin, and ultimately, cell proliferation. Previously, it has been shown that direct activation of G proteins ($G\alpha_i$ and $G\alpha_q$) in vivo with GTPγS in the absence of exogenous Wnt will disrupt the degradation complex and stabilize β-catenin [183]. The viral receptor US28 studied in this thesis, constitutively activates several G protein mediated signal transduction pathways. Therefore, G protein-mediated β-catenin activation could be apparent in HCMV positive cells, expressing constitutively active vGPCRs.