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Title: Interleukin-10s encoded by viruses: a remarkable example of independent acquisitions of a cellular gene by viruses and its subsequent evolution in the viral genome

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Summary

Many viruses have evolved strategies to deregulate the host immune system. These strategies include mechanisms to subvert or recruit the host cytokine network. Interleukin-10 (IL-10) is a pleiotropic cytokine that has both immunostimulatory and immunosuppressive properties. However, its key features relate mainly to its capacity to exert potent immunosuppressive effects. Several viruses have been shown to up regulate the expression of cellular IL-10 (cIL-10), with, in some cases, enhancement of infection by suppression of immune functions. Other viruses encode functional orthologues of cIL-10, called viral IL-10s (vIL-10s). The present review is devoted to these virokines. To date, vIL-10 orthologues have been reported for 12 members of the family *Herpesviridae*, two members of the family *Alloherpesviridae*, and seven members of the family *Poxviridae.* Study of vIL-10s demonstrated several interesting aspects on the origin and the evolution of these viral genes; such as for example, the existence of multiple (potentially up to 9) independent gene acquisition events at different times during evolution, viral gene acquisition resulting from recombination with cellular genomic DNA or cDNA derived from cellular mRNA, and the evolution of cellular sequence in the viral genome to restrict the biological activities of the viral orthologues to those beneficial for the virus life cycle. In this review, various aspects of the vIL-10s described to date are reviewed, including their genetic organization, protein structure, origin, evolution, biological properties and potential in applied research.

1. Introduction

For millions of years, viruses have been co-evolving with their hosts. During this process, they have had to deal with the most complex aspects of host physiology, often mimicking, hijacking or sabotaging host biological processes to their benefit. In this respect, many viruses have evolved strategies to deregulate the host immune response in order to avoid immune surveillance and elimination from the host. These strategies include mechanisms to deregulate the host cytokine network.

The Interleukin (IL)-10 family of cytokines and the related Interferon (IFN) family of cytokines form the larger class II cytokine family (Ouyang *et al.*, 2011). The IL-10 family of cytokines can be categorized into three subgroups, based primarily on biological functions: (i) IL-10 itself; (ii) the IL-20 subfamily cytokines composed of IL-19, IL-20, IL-22, IL-24 and IL-26; and (iii) the type III IFN group (also called IFN λs) (Ouyang *et al.*, 2011; Pestka *et al.*, 2004).

IL-10 is a pleiotropic cytokine, with both immunostimulatory and immunosuppressive properties (Moore *et al.*, 2001). However, its key features relate mainly to its capacity to exert potent effects in the latter category via several mechanisms. Various viruses have been shown to up-regulate the expression of cellular IL-10 (cIL-10), with, in some cases, an enhancement of infection by suppression of immune functions (Brady *et al.*, 2003; Brockman *et al.*, 2009; Díaz-San Segundo *et al.*, 2009; Yu *et al.*, 2008). These studies suggest that cIL-10 expression during the course of infection might be beneficial for the pathogens concerned.

Further supporting this conclusion, several viruses encode orthologues of cIL-10, called viral IL-10s (vIL-10s), that appear to have been acquired by viruses on multiple independent occasions from their host during evolution. This review is devoted to these virokines. Various

aspects of vIL-10 are described, including their genetic organization, protein structure, origin, evolution, biological properties *in vitro* and *in vivo*, and potential in applied research.

2. Discovery of vIL-10s

Cloning and sequencing of the human and mouse IL-10s lead to the identification of the first vIL-10 orthologue. It was discovered that the uncharacterized open reading frame (ORF) BCRF1 of Epstein-Barr virus (EBV; human herpesvirus 4) encodes a protein that exhibited high sequence identity (92.3%) with human IL-10 (Moore *et al.*, 1990). Subsequently, various studies documented that BCRF1 possesses some of the specific biological activities of cIL-10, and it was therefore concluded that this ORF encodes a functional viral orthologue of human IL-10 (Hsu *et al.*, 1990; Niiro *et al.*, 1992). Ever since, the sequencing of an increasing number of viral genomes has revealed a growing list of vIL-10s. To date, vIL-10 orthologues have been reported for 12 members of the family *Herpesviridae*, two members of the family *Alloherpesviridae*, and seven members of the family *Poxviridae* (Table 1).

3. Genetic structure of IL-10 orthologues

The basic structure of the human IL-10 gene consists of five protein-coding exons (I-V) encoding a spliced mRNA of 1629 bp (including untranslated regions) (Fig. 1) (Moore *et al.*, 2001; Sabat, 2010). The first part of exon I and the last part of exon V encode the 5'- and 3'-untranslated regions, respectively. The remaining parts of exons I and V, together with exons II to IV, encode a single protein of 178 amino acid residues. The sizes of the exons are largely conserved among animal species. In contrast, the sizes of the introns show greater variation, and may be up to 1 kbp in length.

The general intron-exon structure of cIL-10 is only found in ovine herpesvirus 2 (OvHV-2) although the introns are considerably shorter than those of its natural host, the sheep (Jayawardane *et al.*, 2008). For the other vIL-10s, variations are observed in the number and positions of introns (Table 1 and Fig. 1) and a large proportion of vIL-10s are intronless.

Viral capture of host genes can result either from recombination between the viral genome and the host genome during viral replication in the nucleus (provided that the viral genome enters the nucleus during replication, as it is the case for herpesviruses but not poxviruses), or from recombination between the viral genome and a retrotranscript (cDNA) of mRNA (Odom *et al.*, 2009; Shackelton & Holmes, 2004). The latter process requires reverse transcriptase activity, most likely derived from retrovirus co-infection of the host cell (Brunovskis & Kung, 1995; Isfort *et al.*, 1992). Direct gene capture from the host genome results in preservation of the original cellular intron-exon structure, as in OvHV-2 (Jayawardane *et al.*, 2008). Subsequent selective pressure could result in successive shortening or even loss of one or more introns, as exemplified by the vIL-10 variants not containing the full subset of exons. The intronless vIL-10 genes most likely represent gene capture via reverse transcription of cellular mRNA, but could theoretically also represent a final stage of intron loss from a gene originally captured from genomic DNA. The fact that all poxvirus vIL-10 genes are intronless probably reflects the cytoplasmic replication cycle of poxviruses, which may exclude the possibility of direct capture of host genes via recombination in the nucleus (Bratke & McLysaght, 2008).

4. Origin and evolution of vIL-10s

Bioinformatical analyses were performed in the context of the present review, firstly to identify all viral sequences encoding IL-10 orthologues that are available in the public databases,

secondly to determine whether these sequences are true vIL-10s or orthologues of cellular genes related to cIL-10. Methods and sequences used for these analyses are provided as supplementary material (S1). The viral sequences listed in Table 1 and the 134R gene encoded by Yaba-like disease virus were detected as viral sequences related to cIL-10 (Lee *et al.*, 2001). Among the sequences listed in Table 1, a sequence highly homologous to EBV vIL-10 was found in the bonobo genome sequence. We assumed that this resulted from the sequencing of a contaminating herpesvirus, hereafter called bonobo herpesvirus (bonobo-HV) (The rational that lead to this conclusion is described in the supplementary material S2). Fig. 2 presents the phylogenetic analysis of all the viral sequences detected above, together with cIL-10 orthologues and representative members of the wider IL-10 family of cytokines. Fig. 2 demonstrates that the 134R protein from Yaba-like disease virus is most closely related to IL-24 proteins, although its exact position in the phylogenetic tree is not well defined in terms of bootstrap values. Further supporting the conclusion that the 134R protein is not an IL-10 orthologue, Barlett *et al.* (2004) demonstrated that it signalled via the IL-20 receptor complex. Thus, it is clear that the 134R protein is not a true vIL-10, and it was therefore removed from further analyses.

Many of the vIL-10 genes are situated in orthologous locations in viral genomes, referred to here as positional orthology. Given that it is unlikely that gene capture would integrate cIL-10 into the same viral genome location on more than one occasion, positional orthology is assumed to represent ancient viral capture events in ancestral viruses. Four positionally orthologous sets of vIL-10 can be defined in the following viral genera: *Cytomegalovirus*, *Lymphocryptovirus*, *Parapoxvirus* and *Capripoxvirus*. All four of these sets cluster together in the Bayesian tree vIL-10s and the cIL-10s of a selection of their hosts (Fig. 3). Based on Fig. 3, it can be concluded that the positionally orthologous clade of vIL-10s of the genus *Lymphocryptovirus*

(EBV/ baboon lymphocryptovirus [BaLCV]/ rhesus lymphocryptovirus [RhLCV]/ bonobo-HV) is nearest neighbour to a clade comprising the corresponding ape cIL-10s. This capture of cIL-10 by an ancestral lymphocryptovirus must therefore have taken place after the divergence of Old World primates from New World primates 42 million years ago, since marmoset IL-10 is an outlier to both members of the genus *Lymphocryptovirus* and Old World primate lineages. The minimum date for this gene capture is more difficult to estimate, as the resolution of the tree does not make it possible to distinguish between it having occurred prior to the human-gorilla divergence, at 9 million years ago, or the ape-monkey divergence, at 29 million years ago. In the vIL-10s of members of the genus *Cytomegalovirus* (HCMV and others in that clade), an ancient capture event can again be inferred because of positional orthology. This event would have to have taken place before 42 million years ago, which is when the Old and New World monkey lineages diverged. Apart from HCMV cmvIL-10, the vIL-10s in the genus *Cytomegalovirus* clade have the same branching pattern as IL-10s of the hosts. The best explanation for the anomalous position of HCMV cmvIL-10 in this clade is that there has been particular selective pressure on HCMV. In this context, it is notable that the nearest relative of HCMV, chimpanzee cytomegalovirus (CCMV), lacks a vIL-10 gene, suggesting that some evolutionary pressure in the common ancestor of humans and chimpanzees resulted in the loss of this gene from CCMV and also its extensive modification in HCMV. Concerning CCMV, as there is only one reported sequence, even if unlikely, one cannot exclude the possibility that this gene has been lost during viral replication in cell culture. For the positionally orthologous vIL-10s of members of the genus *Parapoxvirus* (orf virus [ORFV]/ bovine papular stomatitis virus [BPSV]/ pseudocowpox virus [PCPV]), it is apparent that the ancestor of these proteins was captured prior to divergence of the sheep and goat lineages at 7.3 million years ago. However, it is more difficult to specify a

maximum date for this gene capture event, since the relationships of parapoxvirus vIL-10s to bovine and cervine IL-10 are poorly resolved.

The Bayesian tree does not help with the assessment of the vIL-10s of the fish viruses anguillid herpesvirus 1 (AngHV-1) and cyprinid herpesvirus 3 (CyHV-3), nor of OvHV-2, canarypox virus (CNPV) or the capripoxviruses (lumpy skin disease virus [LSDV]/ sheeppox virus [SPV]/ goatpox virus [GPV]). However, it seems unlikely that any of these vIL-10s represents a recent capture from the host. The capripoxvirus vIL-10s constitute a clade, but its point of divergence from the host sequences cannot be pinpointed in the same way as for the parapoxviruses. The only obvious example of a recent gene capture event for the origin of a vIL-10 is in equid herpesvirus 2 (EHV-2).

Overall, based on positional orthology, amino acid sequence comparisons and the presumed modes of gene capture, at least eight, and possibly nine (assuming that AngHV-1 and CyHV-3 vIL-10s represent independent acquisitions), different viral cIL-10 capture events can be discriminated.

5. Protein structure of IL-10 orthologues

Amino acid sequence conservation is rather low among the three subgroups of the IL-10 family of cytokines (IL-10, IL-20 subfamily cytokines and type III IFN group) (Zdanov, 2004). In particular, type III IFNs are closer to type I IFNs than to the IL-10. For example, the amino acid sequence of IFN-λ3 (which belongs to the type III IFN group) is more similar to that of type I IFNs (exhibits 33% of similarity) than to the IL-10 (exhibits 23% of similarity) (Gad *et al.*, 2009). Moreover, induction of gene expression and biological activities of type III IFNs are more similar to those described for type I IFNs (Ouyang *et al.*, 2011). However, IFN-λ3 is structurally

more closely related to the IL-10 family of cytokines, especially IL-22 (Gad *et al*., 2009) and has been shown to signal through the same IL-10R2 chain (Ouyang *et al*., 2011).

Cellular IL-10s are well conserved among species (Lockridge *et al.*, 2000; Moore *et al.*, 2001). Indeed, the high level of conservation among cIL-10s contrasts with the variable (25-97.2%), and frequently low levels of identity observed between vIL-10s and their respective host IL-10s (Table 1). However, as illustrated in Fig. 1 (colour code) the percentage of conservation is not distributed uniformly along vIL-10s. It is generally higher for amino acid regions corresponding to the regions encoded by cIL-10 exons 1 (with exception of the signal peptide region), 3 and 5.

Independent of the level of identity between vIL-10s and cIL-10s, the former share many features with the latter. Firstly, cIL-10s and vIL-10s are secreted proteins. They are synthesized as precursors expressing a 17-33 residue hydrophobic signal peptide at the N terminus (Table 1). This peptide is cleaved during secretion (Kotenko & Pestka, 2001). Secondly, all cIL-10s encode two family signature motifs: L-[FILMV]-X3-[ILV]-X3-[FILMV]-X5-C-X5-[ILMV]-[ILMV]-X(3)-L-X2-[IV]-[FILMV] and KA-X2-E-X-D-[ILV]-[FLY]-[FILMV]-X2-[ILMV]-[EKQZ] (Pinto *et al.*, 2007; Zhang *et al.*, 2005). These motifs, which are essential for the structure and the function of cIL-10s, are conserved to a large extent in vIL-10s. Thirdly, despite the variable sequence homology observed between cIL-10s and vIL-10s at the amino acid sequence level, their determined or predicted structures are highly conserved (Fig. 4). The crystal structure of human IL-10 has been determined as free ligand (Walter & Nagabhushan, 1995; Yoon *et al.*, 2006; Zdanov *et al.*, 1995; Zdanov *et al.*, 1996) and as a binary complex bound to its soluble receptor (Josephson et al., 2001). These studies demonstrated that cIL-10, like all members of the IL-10 family of class II cytokines, possesses a characteristic α-helical fold consisting of six

helices (A to F) and connecting loops (Fig. 4a). It is secreted as a domain-swapped homodimer in which two adjacent non-covalently bounded peptides exchange helices E and F to form a twofold symmetric, V-shaped reciprocal dimer (Zdanov *et al.*, 1995). The crystal structures of EBV and HCMV cmvIL-10 have been determined (Jones *et al.*, 2002; Yoon *et al.*, 2005; Zdanov *et al.*, 1997) (Fig. 4c and d), and were proved to be similar to that of human IL-10 with exception that HCMV cmvIL-10 lacks helix B (Jones *et al.*, 2002). Using the receptor-bound structure of human IL-10 as template (Josephson *et al.*, 2001), the three-dimensional protein structures of the CyHV-3 and AngHV-1 vIL-10s and the cIL-10s of their respective host were predicted (van Beurden *et al.*, 2011) (Fig. 4e and f). These *in silico* analyses suggested that the vIL-10s encoded by these two alloherpesviruses share the conserved structure described for cIL-10.

6. Transcriptomic and proteomic expression of vIL-10 genes

Expression of vIL-10 genes has been studied at the RNA and protein levels. Depending on the viral species, genes encoding vIL-10s have been shown to be transcribed during *in vitro* replication at early times for rhesus cytomegalovirus (RhCMV) (Lockridge *et al.*, 2000), HCMV LAcmvIL-10 (Jenkins *et al.*, 2008a) and CyHV-3 (Ilouze *et al.*, 2012), at early-late times for CyHV-3 (Ouyang *et al.*, 2013), or at late times for EBV (Hudson *et al.*, 1985; Miyazaki *et al.*, 1993; Touitou *et al.*, 1996), HCMV cmvIL-10 (Chang *et al.*, 2004) and AngHV-1 (van Beurden *et al.*, 2013).

The HCMV UL111A gene encodes a vIL-10 and has been shown to generate different transcripts during lytic and latent infections as a consequence of differential splicing (Kotenko *et al.*, 2000; Jenkins *et al.*, 2004). HCMV cmvIL-10 is expressed during the productive phase of infection (Spencer *et al.*, 2002; Chang *et al.*, 2004), whereas LAcmvIL-10 has been reported to

be expressed during both latent (Jenkins *et al.*, 2004) and productive infections (Jenkins et al., 2008a). Both transcripts share the same initiation codon. However, as a result of the lack of splicing of the second intron, LAcmvIL-10 retains only the first two exons present in the lytic transcript (cmvIL-10), resulting in an in-frame stop codon 12 codons after the second exon. As a consequence, LAcmvIL-10 encodes a truncated protein of 139 residues that shares its first 127 residues with the longer protein encoded by the cmvIL-10 transcript (Jenkins *et al.*, 2004). Also, Lin *et al.* (2008) described five cmvIL-10 isoforms resulting from alternative splicing during *in vitro* replication of HCMV (Lin *et al.*, 2008).

EBV BCRF1 was classified as a late gene (Hudson *et al.*, 1985), although it is expressed in B cells relatively early after infection (Jochum *et al.*, 2012; Miyazaki *et al.*, 1993). There is no evidence for BCRF1 transcription and protein secretion during *in vitro* latency. However, in *in vivo* studies, Xu *et al.* (2001) detected expression of BCRF1 in latently infected patients with NK/T-cell lymphoma (Xu *et al.*, 2001).

Expression of CyHV-3 ORF134, which encodes a vIL-10, has been detected *in vivo* during acute primary infection and subsequent reactivation phases. Expression during persistent infection at restrictive temperature was low or below the detection level (Sunarto *et al.*, 2012).

Secretion of vIL-10 in the extracellular compartment has been demonstrated for several viruses in cell culture: RhCMV (Lockridge *et al.*, 2000), HCMV (cmvIL-10) (Chang *et al.*, 2004), EBV (Touitou *et al.*, 1996) and CyHV-3 (Ouyang *et al.*, 2013). *In vivo* secretion has been demonstrated for RhCMV (Lockridge *et al.*, 2000).

The effect of vIL-10 on virus growth *in vitro* has been studied using recombinant strains containing knock-out or nonsense mutations. For all viruses tested, vIL-10 genes were shown to

be non-essential for growth of HCMV (Dunn *et al.*, 2003), RhCMV (Chang & Barry, 2010), EBV (Jochum *et al.*, 2012), CyHV-3 (Ouyang *et al.*, 2013) and ORFV (Fleming *et al.*, 2007).

7. Ligand-receptor complexes formed by IL-10 orthologues

Cellular IL-10 acts through a specific cell surface receptor (IL-10R) complex, which is composed of two different class II cytokine receptor family (CRF2) subunits, IL-10R1 and IL-10R2 (Moore *et al.*, 2001; Zdanov, 2004). IL-10R1 is the high-affinity receptor subunit of cIL-10 and is expressed mainly on immune cells (Liu *et al.*, 1994). Cellular IL-10 first binds to IL-10R1, which leads to changes of its conformation and subsequent association with the low-affinity receptor subunit IL-10R2 (Yoon *et al.*, 2006). In contrast to IL-10R1, IL-10R2 has a broader expression pattern, being expressed on most immune and non-immune cells. However, IL-10R2 is unable to bind cIL-10 in the absence of IL-10R1 (Kotenko *et al.*, 1997; Wolk *et al.*, 2004). Binding of cIL-10 to the IL-10R complex activates a signalling pathway, which mainly acts through receptor-associated Janus kinase 1 (Jak1, associated with IL-10R1), Tyrosine kinase 2 (Tyk2, associated with IL-10R2) and Signal transduction and transcription (STAT) factors, leading to initiation of transcription of the appropriate genes (Sabat *et al.*, 2010).

HCMV cmvIL-10 and EBV vIL-10 have been shown to bind to and signal through human IL-10R1 (Jones *et al.*, 2002; Yoon *et al.*, 2005). The regions of the surfaces of the human IL-10 and vIL-10 variants that make contact with the receptor are essentially the same. The binding affinity of HCMV cmvIL-10 (which exhibits only 27% sequence similarity with human IL-10) to soluble IL10R1 (sIL10 R1) is essentially similar to that of human IL-10 (Jones *et al.*, 2002). Furthermore, HCMV cmvIL-10 induces phosphorylation of the transcription factor STAT3 in monocytes, indicating its ability to bind and signal through human IL-10R in a manner

comparable to that of human IL-10 (Jenkins *et al*., 2008b). The same authors blocked the ability of cmvIL-10 to down-regulate the MHC class II expression on monocytes by using neutralizing antibodies raised against human IL-10R (Jenkins *et al*., 2008b). None of the five cmvIL-10 isoforms resulting from alternative splicing during *in vitro* replication of HCMV induced phosphorylation of STAT 3 despite being able to bind to human IL-10R (Lin *et al.*, 2008). In contrast to cmvIL-10, LAcmvIL-10 does not induce STAT3 phosphorylation and retains the ability to reduce MHC class II expression on monocytes in the presence of neutralizing antibodies raised against human IL-10R (Jenkins *et al*., 2008b). These results suggest that LAcmvIL-10 does not bind to human IL-10R or acts through another receptor or binds to human IL-10R but in a different way as compared to cmvIL-10 and human IL-10. These variations most probably resulted from the fact that LAcmvIL-10 is a truncated protein that lacks C-terminal helices E and F. As a consequence LAcmvIL-10 lacks many of immunosuppressive functions (see below) that are known for cmvIL-10 (Jenkins *et al*., 2008b).

The most prominent structural difference between human IL-10 and HCMV cmvIL-10 bound to sIL-10R1 is the ~40º interdomain angle, which forces a reorganization of the IL-10R1 subunits in the putative cell surface complex (Jones *et al.*, 2002). The binding affinity of EBV vIL-10 (which has 92% sequence identity to human IL-10) to cell surface IL-10R1 is approximately a thousand-fold lower than that of human IL-10 (Liu *et al.*, 1997). This difference in receptor binding affinity is thought to be caused by subtle changes in the conformation and dynamics of two loop structures and the interdomain angle (Yoon *et al.*, 2005), as well as by single amino acid substitutions (Ding *et al.*, 2000).

Because the crystal structures of human IL-10 and EBV vIL-10 are very similar, the observed functional differences (described below) have been attributed to differences in binding

affinity (Ding *et al.*, 2001; Liu *et al.*, 1997). Recently, the biological effect induced by CyHV-3 vIL-10 in zebrafish embryos was shown to be abrogated by down-regulation of IL-10R1 expression using a specific morpholino, suggesting that CyHV-3 vIL-10 functions also through IL-10R1 (Sunarto *et al.*, 2012).

8. Biological activities of IL-10 orthologues

8.1 Biological activities of cIL-10

Cellular IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), an immune mediator that is produced by Th2 cell clones and has inhibitory effects on the synthesis of IL-2 and IFN-γ by Th1 cell clones (Fiorentino *et al.*, 1989). Today, it is known that many different myeloid and lymphoid cells have the ability to produce IL-10 (Couper *et al.*, 2008; Mosser & Zhang, 2008; Sabat *et al.*, 2010), and that infection by a single pathogen species induces secretion of cIL-10 by more than one cell population, depending on the type of pathogen, the infected tissue and the time point in the immune response (Sabat *et al.*, 2010).

Cellular IL-10 is a type II pleiotropic cytokine with both immunostimulatory and immunosuppressive properties (Moore *et al.*, 2001). However, the key features of this cytokine relate to its capacity to exert potent immunosuppressive functions on several immune cell types (Moore *et al.*, 1993). It shows a clear, direct immunosuppressive effect on activated monocytes/macrophages, both by inhibition of the release of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, IL-8, granulocyte colony-stimulating factor [G-CSF] and granulocyte-macrophage colony-stimulating factor [GM-CSF]) (de Waal Malefyt *et al.*, 1991a; Fiorentino *et al.*, 1991) and by enhancing the release of anti-inflammatory mediators (such as

IL-1 receptor antagonist and soluble TNF-α receptor) (Hart *et al.*, 1996; Jenkins *et al.*, 1994). Additionally, cIL-10 inhibits antigen presentation by down-regulation of the expression of MHC class I, MHC class II and B7-1/B7-2 co-stimulatory molecules (de Waal Malefyt *et al.*, 1991b; Matsuda *et al.*, 1994; Willems *et al.*, 1994). It also affects dendritic cells (DCs) by preventing their differentiation from monocyte precursors, and their maturation (Allavena *et al.*, 1998; Demangel *et al.*, 2002). Furthermore, cIL-10 hampers the development of Th1 immunity, both indirectly by inhibiting IL-12 synthesis by antigen presenting cells (APCs) and directly by inhibiting IL-2 and IFN-γ production by Th1 cells (D'Andrea *et al.*, 1993; Fiorentino *et al.*, 1991). Moreover, cIL-10 acts directly on Th2 cells and inhibits IL-4 and IL-5 synthesis (Del Prete *et al.*, 1993). Cellular IL-10 has also immunosuppressive effect on neutrophilic and eosinophilic granulocytes by preventing the synthesis of lipopolysaccaride (LPS)-induced pro-inflammatory mediators (Cassatella *et al.*, 1993; Takanaski *et al.*, 1994). Thus, cIL-10 plays a key role in the inhibition of the pro-inflammatory responses. It is thought that the role of this inhibition is to protect tissues from the lesions that could result from exaggerated inflammation (Banchereau *et al.*, 2012).

Notably, apart from its immunosuppressive role, cIL-10 also shows a stimulatory effect on several types of immune cell. It may prevent apoptosis of B cells, enhancing their activation and contributes to immunoglobulin class switching (Go *et al.*, 1990; Rousset *et al.*, 1992). Cellular IL-10 alone or in combination with other cytokines may also have a stimulatory effect on proliferation of, and cytokine production by, certain subsets of cytotoxic T cells (Rowbottom *et al.*, 1999; Santin *et al.*, 2000), mast cells (Thompson-Snipes *et al.*, 1991) and NK cells (Cai *et al.*, 1999; Carson *et al.*, 1995).

8.2. Biological activities of vIL-10s

The biological activities of vIL-10s have been studied mainly *in vitro* using recombinant proteins generated from bacterial or mammalian cell expression systems, supernatants from viral infected cultures, or, to a lesser extent, recombinant vIL-10 knock-out viruses. Only a restricted number of studies have addressed the roles of vIL-10s *in vivo* by comparing wild type and vIL-10 knock-out viruses. These *in vitro* and *in vivo* studies are summarized below. *In vitro* studies are presented according to the immune process affected by vIL-10s, while *in vivo* studies are organized per virus species studied.

8.2.1 Biological activities of vIL-10s determined *in vitro*

8.2.1.1 Inhibition of cytokine synthesis and leukocyte proliferation.

The hallmark activity of cIL-10 is the inhibition of cytokine production following pro-inflammatory signals. *In vitro* studies suggest that this activity is conserved among most viral orthologues. The studies supporting this conclusion are summarized below.

HCMV cmvIL-10 inhibits gene expression and secretion of pro-inflammatory cytokines by LPS-stimulated peripheral blood mononuclear cells (PBMCs), monocytes, monocyte-derived dendritic cells (MDDCs) and plasmacytoid dendritic cells (PDCs) (Avdic *et al.*, 2013; Chang *et al.*, 2009; Chang *et al.*, 2004; Jenkins *et al.*, 2008b; Nachtwey & Spencer, 2008; Raftery *et al.*, 2004; Spencer, 2007; Spencer *et al.*, 2002). Similarly, the orthologous RhCMV vIL-10 has been shown to inhibit production of pro-inflammatory cytokines by LPS-stimulated PBMCs and monocytes (Logsdon *et al.*, 2011; Spencer *et al.*, 2002). In addition, both HCMV cmvIL-10 and RhCMV vIL-10 reduced IFN-γ production by PHA-stimulated human PBMCs, as well as human and rhesus PBMC proliferation (Spencer *et al.*, 2002). HCMV cmvIL-10 secreted by

HCMV-infected cells can directly suppress the synthesis of type I IFNs by plasmacytoid dendritic cells (PDCs) (Chang *et al.*, 2009), demonstrating that HCMV cmvIL-10 can act in *trans*, since PDCs are highly resistant to infection by HCMV (Slobedman *et al.*, 2009). HCMV cmvIL-10 has a marked impact on microglial cells, which play a role in host defense against HCMV brain infection. Pretreatment of microglial cells with recombinant HCMV cmvIL-10 prior to stimulation with HCMV significantly decreased the protein level of CXC chemokine ligand 10 (CXCL10), which is known to be involved in the recruitment of activated T lymphocytes in infected tissues (Cheeran *et al.*, 2003). Very recent studies demonstrated that cmvIL-10 influence monocyte polarization by induction of development of M2 alternatively activated monocytes type c (M2c). The M2c polarization of monocytes by cmvIL-10 resulted in up-regulation of the anti-inflammatory enzyme heme oxygenase 1 (HO-1), and this was shown to play an important role in viral IL-10-mediated suppression of pro-inflammatory cytokines by M2c monocytes (Avdic *et al*., 2013). Moreover, M2c monocyte polarization by cmvIL-10 reduces the ability to stimulate $CD4^+$ T cell activation and proliferation (Avdic *et al*., 2013).

In contrast to cmvIL-10, LAcmvIL-10 showed no inhibitory effect on IL-1α, IL-1β, IL-6 or TNF-α expression by LPS-stimulated MDDCs (Jenkins *et al.*, 2008b). However, in another study, it has been shown to inhibit TNF-α production by THP-1 myeloid cells stimulated with LPS (Spencer *et al.*, 2008). Finally, Avdic *et al.* (2011) demonstrated significantly higher levels of transcription and secretion of cytokines associated with DC formation, as well as an increase in the proportion of myeloid DCs in $CD34^+$ primary myeloid progenitor cells latently infected with HCMV deleted for the UL111A gene region, compared to parental virus or mock infection (Avdic *et al.*, 2011).

EBV vIL-10 inhibits pro-inflammatory cytokine production by activated cells of various types (de Waal Malefyt *et al.*, 1991a; Hsu *et al.*, 1990; Jochum *et al.*, 2012; Salek-Ardakani *et al.*, 2002b; Vieira *et al.*, 1991). In addition, it reduces both the amount of IFN-γ mRNA (Niiro *et al.*, 1992) and IFN-γ secretion (Salek-Ardakani *et al.*, 2002b) in activated human PBMCs. Jochum *et al.*, 2012 demonstrated that human PBMCs infected with EBV deleted for BCRF1 produced significantly higher levels of the pro-inflammatory cytokines IFN-γ, IL-2, IL-6 and TNF-β, whereas levels of IL-1, IL-5, IL-8 and TNF-α were similar to those observed with the parental wild type strain. Interestingly, these authors also observed an increased production of human IL-10 by PBMCs infected with the BCRF1deleted strain. This observation suggests that vIL-10 could regulate human IL-10 expression. However, the observed effect could also have been an indirect consequence of the higher level of pro-inflammatory cytokines resulting from infection by the EBV vIL-10-deleted recombinant (Jochum *et al.*, 2012). Finally, Brodeur and Spencer (2010) demonstrated that anti-human IL-10 antibodies bind to and neutralize the immunosuppressive activity of EBV vIL-10 but not HCMV cmvIL-10. This observation is consistent with the higher homology existing between EBV vIL-10 and human IL-10 (92.3% of identity) compared to HCMV cmvIL-10/ human IL-10 (27.3% of identity).

The inhibition of cytokine activities were also demonstrated for two viruses infecting sheep (OvHV2 and ORFV) using different *in vitro* systems. OvHV2 vIL-10 inhibited IL-8 production by LPS-stimulated ovine macrophages (Jayawardane *et al.*, 2008) whereas ORFV vIL-10 inhibited TNF-α and IL-8 production from LPS-stimulated ovine macrophages and ionophore/PMA stimulated keratinocytes, as well as IFN-γ and GM-CSF production by Con-A-stimulated PBMCs (Haig *et al.*, 2002a, b). However, ORFV vIL-10 knock-out virus showed no effect on infected keratinocyte IL-8 and TNF-α production (Haig *et al.*, 2002b).

ORFV vIL-10 has also been shown to inhibit expression and secretion of TNF-α in LPS-activated mouse peritoneal macrophages (Imlach *et al.*, 2002), to inhibit TNF-α and IL-1β in the human monocyte cell line THP-1 activated by LPS (Imlach *et al.*, 2002; Wise *et al.*, 2007), and to inhibit production of IL-8, IL-1β and TNF-α in LPS-stimulated ovine alveolar macrophages (Fleming *et al.*, 2000). Furthermore, inhibition of IFN-γ production in PBMCs by ORFV vIL-10 was demonstrated (Fleming *et al.*, 2000). Compared to human IL-10, ORFV vIL-10 possesses reduced ability to impair THP-1 monocyte proliferation in the presence of LPS (Wise *et al.*, 2007). However, it would be interesting to compare the biological activities of ORFV vIL-10 to those of ovine IL-10.

8.2.1.2 Deregulation of MHC and co-stimulatory molecule expression

Studies of the vIL-10s encoded by HCMV and EBV have demonstrated their ability to deregulate MHC and co-stimulatory molecule expression. HCMV cmvIL-10 and RhCMV vIL-10 reduced cell surface expression of classical MHC class I and class II molecules (Jaworowski *et al.*, 2009; Jenkins *et al.*, 2008b; Raftery *et al.*, 2004; Spencer *et al.*, 2002), but also increased expression of the non-classical MHC molecules HLA-DM and HLA-G on LPS-stimulated human MDDCs and monocytes, respectively (Raftery *et al.*, 2004; Spencer *et al.*, 2002). These observations suggest that HCMV cmvIL-10 could prevent antigen presentation to T cells through MHC class I molecule down-regulation but could simultaneously protect MHC class I-negative cells from NK cell-mediated lysis through up-regulation of HLA-G (Rouas-Freiss *et al.*, 1997). Although independent studies demonstrated the inhibitory effect of HCMV cmvIL-10 on MHC class I expression in different LPS-stimulated cell types, Pepperl-Klindworth *et al*. (2006) suggested that HCMV cmvIL-10 secreted during the productive phase

of HCMV infection has no direct impact on MHC class I-restricted antigen presentation on non-infected bystander cells in the context of viral infection (Pepperl-Klindworth *et al.*, 2006). HCMV cmvIL-10 has also been shown to inhibit LPS-induced enhancement of co-stimulatory molecules (CD40, CD80, CD86, B7-H1 and B7-DC) on the surface of MDDCs (Jenkins *et al.*, 2008b; Raftery *et al.*, 2004). LAcmvIL-10 reduces the expression of MHC class II molecules, but, in contrast to cmvIL-10, does not down-regulate expression of MHC class I molecules and co-stimulatory molecules (CD40, CD80, and CD86) on LPS-stimulated MDDCs (Jenkins *et al.*, 2008b). The reduction of cell surface MHC class II molecule expression by LAcmvIL-10 was comparable to the effect of cmvIL-10 both on immature myeloid progenitor cells and human monocytes (Jaworowski *et al.*, 2009; Jenkins *et al.*, 2008b). Jenkins *et al.* (2008b) suggested a possible mechanism for the reduction of MHC class II cell surface expression at the level of the transcriptional activity of CIITA, a gene that encodes a protein regulating the transcription of genes involved in the MHC class II biosynthesis pathway. The authors demonstrated that cmvIL-10, as well as LAcmvIL-10, significantly inhibited transcription of CIITA, and that this resulted in down-regulation of expression of HLA-DR α, β and invariant chain. In addition, both cmvIL-10 and LAcmvIL-10 may inhibit MHC class II surface expression acting at the post-translational level by blocking transport of MHC class II molecules to the cell surface (Jenkins *et al.*, 2008b). In addition to the above-mentioned functional studies utilizing recombinant LAcmvIL-10, Cheung *et al.* (2009) demonstrated that $CD34^+$ myeloid progenitor cells latently infected by an HCMV strain deleted for the UL111A gene expressed a higher level of surface MHC class II molecules compared to cells infected with the parental strain. Cells infected with the knock-out strain became recognizable by allogeneic and autologous $CD4^+$ T cells (Cheung *et al.*, 2009).

EBV vIL-10 was shown to reduce both constitutive and IFN-γ- or IL-4-induced MHC class II cell surface expression on monocytes and macrophages (de Waal Malefyt *et al.*, 1991b; Salek-Ardakani *et al.*, 2002a, b). This resulted in a decrease of antigen presentation by monocytes, and, as a consequence, a reduction of T cell proliferation (de Waal Malefyt *et al.*, 1991b). EBV vIL-10 also inhibited the expression of adhesion molecule ICAM-1 and co-stimulatory molecules (CD80 and CD86) on monocytes and macrophages when added simultaneously with IFN-γ (Salek-Ardakani *et al.*, 2002a). Interestingly, EBV vIL-10 inhibited IFN-γ-induced MHC class I expression on monocytes and macrophages only when it was added 2 h prior to the addition of IFN-γ, suggesting that it affects an early step in the IFN-γ signalling pathway (Salek-Ardakani *et al.*, 2002a).

8.2.1.3. Inhibition of DC

Dendritic cells play key roles in immune responses. Viral IL-10s have been shown to affect their maturation, functionality and survival. HCMV cmvIL-10 inhibited LPS-induced pro-inflammatory cytokine production by immature DCs (Chang *et al.*, 2004; Raftery *et al.*, 2008), but was also shown to have pronounced long-term effects on mature DCs. Although it enhanced the migration of mature DCs towards peripheral lymph nodes, it also reduced their production of cytokine (Chang *et al.*, 2004). In addition, the inability of mature DCs to secrete IL-12 was maintained, even when they were restimulated by the activated T-cell signal CD40 ligand in the absence of cmvIL-10. Finally, cmvIL-10 induced endogenous cIL-10 expression in DCs, further increasing its modulatory effects (Chang *et al.*, 2004).

Raftery *et al.* (2004) demonstrated that HCMV cmvIL-10, in contrast to EBV vIL-10, had additional effects on DCs that could affect negatively their roles in immunity. Firstly, it inhibited

cell-surface expression of molecules involved in antigen presentation, co-stimulation and adhesion. Secondly, it increased apoptosis of LPS-stimulated immature DCs by blocking expression of the anti-apoptotic, long form cellular FLIP protein. Thirdly, it induced a strong activation of STAT3 (a key mediator in cIL-10 transduction signal) in immature DCs. Fourthly, it up-regulated expression of DC-SIGN and IDO on LPS-stimulated immature DCs (Raftery *et al.*, 2004). DC-SIGN has been shown to play a role in DC infection with primary HCMV isolates (Halary *et al.*, 2002), whereas synthesis of IDO by human DCs caused suppression of T cell responses (Hwu *et al.*, 2000). In contrast to HCMV cmvIL-10, LAcmvIL-10 showed no inhibitory effect in LPS-stimulated immature DCs on the expression of pro-inflammatory cytokines, co-stimulatory molecules and the maturation marker CD83 (Jenkins *et al.*, 2008b). However, using a recombinant virus deleted for the UL111A gene region, Avdic *et al.* (2011) demonstrated that HCMV vIL-10 expressed during latency inhibits differentiation of latently infected myeloid progenitor cells toward a DC phenotype, suggesting that LAcmvIL-10 may inhibit infected myeloid progenitors to differentiate into DCs, thereby limiting the presentation of latency-associated viral peptides by DCs (Avdic *et al.*, 2011).

Immature DCs exposed simultaneously to LPS and ORFV vIL-10 showed enhanced ovalbumin–FITC uptake and reduced IL-12 expression, indicating inhibition of maturation of DCs. Furthermore, ORFV vIL-10 inhibited the up-regulation of DC cell-surface markers of activation and maturation such as MHC class II, CD80, CD83 and CD86, and inhibited the capacity of DCs to activate $CD4^+$ T cells (Chan *et al.*, 2006). Similarly, ORFV vIL-10 inhibited maturation and expression of MHC class II, CD80 and CD86 in stimulated murine bone marrow-derived dendritic cells (BMDCs), and reduced their ability to present antigens (Lateef *et al.*, 2003).

8.2.1.4. Other immunosuppressive properties

In addition to the main immunosuppressive properties described above, some studies suggest potential additional immunosuppressive effects for some vIL-10s. HCMV cmvIL-10 decreased matrix metalloproteinase activity and deregulated cell-to-cell or cell-matrix interactions of infected cytotrophoblasts and endothelial cells (Yamamoto-Tabata *et al.*, 2004). EBV vIL-10 has been shown to impair some of the defense mechanisms of activated monocytes and macrophages. It inhibited production of the superoxide anion by PBMCs and monocytes (Niiro *et al.*, 1992) and PGE2 expression by LPS-stimulated monocytes (Niiro *et al.*, 1994). Furthermore, EBV vIL-10 inhibits NK/NKT cell-mediated lysis of infected B cells through a direct effect on these cytotoxic cells and also through an indirect inhibitory effect on the CD4+ T cells that contribute to the microenvironment required for NK/NKT cytotoxicity (Jochum *et al.*, 2012).

8.2.1.5. Immunostimulatory properties

In addition to their immunosuppressive effects, some vIL-10s have retained at least some of the immunostimulatory properties of their cellular orthologues. HCMV cmvIL-10, but not LAcmvIL-10, showed a strong stimulatory effect on proliferation of the human B cell lymphoma Daudi cell line (Spencer *et al.*, 2008) and induced the production of human IL-10 (which is a growth factor for B lymphocytes) (Jaworowski *et al.*, 2009; Spencer *et al.*, 2008). Jaworowski *et al.* (2009) studied the effect of cmvIL-10 and LAcmvIL-10 on monocytes. They demonstrated that cmvIL-10 but not LAcmvIL-10 increases the expression of Fcγ receptors CD32 and CD64, as well as Fcγ-receptor-mediated phagocytosis (Jaworowski *et al.*, 2009). RhCMV vIL-10 has

been shown to stimulate proliferation of TF-1/IL-10R1 cells, which are human erythroleukemic cells proliferating upon addition of human IL-10 to the media (Logsdon *et al.*, 2011).

EBV vIL-10 has also been shown to stimulate proliferation and differentiation of human B cells as well as immunoglobulin production (Defrance *et al.*, 1992; Rousset *et al.*, 1992; Stuart *et al.*, 1995). However, EBV vIL-10 lacks several of the other immunostimulatory functions expressed by cIL-10, such as co-stimulation of mouse thymocyte proliferation, mast cell proliferation and up-regulation of MHC class II expression on B cells (Vieira *et al.*, 1991).

The ability of the OvHV-2 and ORFV vIL-10s to stimulate cell proliferation to levels comparable to those obtained with ovine IL-10 has been demonstrated by independent studies. OvHV-2 vIL-10 induced proliferation of murine mast cell line D-36 in conjunction with IL-4 (Jayawardane *et al.*, 2008). ORFV vIL-10 has been shown to induce proliferation of murine thymocytes in the presence of IL-2 (Fleming *et al.*, 1997), ovine mast cells stimulated with IL-3, murine mast cell line D-36 stimulated with IL-4 (Haig *et al.*, 2002b) and murine MC/9 mast cells stimulated with IL-3 and IL-4 (Imlach *et al.*, 2002).

8.2.2 Biological activities of vIL-10s determined *in vivo*

Numerous molecular and *in vitro* studies suggest that, following capture, there has been adaptive evolution of vIL-10 through positive selection to retain the properties most beneficial for the viral life cycle. However, very few studies have addressed the role of vIL-10 *in vivo* by comparison of a wild type strain and derived deleted and revertant strains. This approach, which is essential to drawing conclusions on biological relevance *in vivo*, has been followed for only three viruses: RhCMV, ORFV and CyHV-3.

Chang and Barry (2010) demonstrated that RhCMV vIL-10 has various effects on both the innate and the adaptive immune responses against RhCMV in infected rhesus macaques. They performed comparative infections with a wild type strain and a derived recombinant strain deleted for UL111A. Skin biopsies from macaques infected with the deleted strain exhibited a higher level of cellularity at the site of infection but contained a lower frequency of $CD68^+$ macrophages. The latter observation suggests that RhCMV vIL-10 could contribute to the recruitment of permissive cells on viral replication sites. RhCMV vIL-10 was also shown to reduce trafficking of myeloid DCs to draining lymph nodes and to decrease priming of naïve $CD4^+$ T cells (Chang & Barry, 2010). Although RhCMV vIL-10 has no effect on IgM production, it inhibited B cell differentiation and antibody isotype switching, resulting in a permanent deficit of circulating anti-RhCMV IgG. In addition, RhCMV vIL-10 delayed antibody maturation and attenuated the magnitude of anti-viral antibody titre (Chang & Barry, 2010). Finally, it was also shown to reduce the frequency of RhCMV-specific effector T helper cells secreting IFNγ or IL-2, and T cell proliferation (Chang & Barry, 2010).

The activity of vIL-10 encoded by ORFV *in vivo* has been analyzed in its natural host, the sheep. A preliminary study revealed that the frequency of IFNγ mRNA-expressing cells in skin lesions was higher in animals infected with the vIL-10 knock-out virus than in animals infected with the parental wild type virus (Fleming *et al.*, 2000). Interestingly, after primary infection, smaller, less severe lesions were observed in animals infected with the vIL-10 knock-out virus than those observed in animals infected with the wild type parental or revertant strains (Fleming *et al.*, 2007).

Recently, the role of CyHV-3 vIL-10 was studied *in vivo* using an artificial zebrafish embryo model (Sunarto *et al.*, 2012). It was shown that injection of CyHV-3 ORF134 mRNA

into zebrafish embryos increased the number of lysozyme-positive cells to a degree similar to that of zebrafish IL-10 mRNA (Sunarto *et al.*, 2012). However, Ouyang *et al.* (Ouyang *et al.*, 2013) demonstrated that CyHV-3 vIL-10 does not significantly affect its virulence in common carp or the host innate immune response. Thus, infection of carp with ORF134-deleted, ORF134-revertant or wild type strains induced comparable levels of CyHV-3 disease (Ouyang *et al.*, 2013). Moreover, quantification of viral load and real-time PCR investigating the expression of several carp inflammatory cytokines at various times post-infection did not revealed any significant differences between groups of fish infected with the three viral genotypes (Ouyang *et al.*, 2013). Similarly, histological examination of the gills and the kidneys of infected fish revealed no significant differences between fish infected with the ORF134-deleted virus and those infected with the control parental or revertant strains (Ouyang *et al.*, 2013). All together, the results demonstrated that CyHV-3 vIL-10 is essential for neither viral replication *in vitro* nor virulence in common carp.

9. Viral IL-10s as a topic of applied research

In addition to their importance in fundamental research, a large number of studies demonstrate a role for vIL-10s in applied research. A thorough description of this abundant literature is beyond the scope of this review. Here, we briefly describe the two main types of applied research developed on vIL-10s. These studies investigate the potential of vIL-10s as candidate antigens or target genes (production of attenuated recombinant vaccines) for the development of anti-viral vaccine or as an immunosuppressor to prevent immunopathologies.

For vIL-10s that alter innate or adaptive immunity *in vivo*, vaccine-mediated neutralization of their function could contribute to inhibition of the establishment of a persistent

infection in naïve subjects or even interrupt a pre-existing persistent infection. This theoretical possibility could apply to most vIL-10s that are quite divergent in sequence from the host IL-10. To address this concept using the RhCMV model (Yue & Barry, 2008), inactive RhCMV vIL-10 mutants were designed as antigen candidates and shown to induce the production of neutralizing antibodies specific to vIL-10 (not cross-reacting with host IL-10) (de Lemos Rieper *et al.*, 2011; Logsdon *et al.*, 2011). The ability of such an antigen candidate to interfere with persistent RhCMV infection (establishment or maintenance) has not yet been tested. However, a recent study on the immunogenicity of vIL-10 in RhCMV-infected rhesus macaques demonstrated that the serum of persistently infected animals contains high levels of vIL-10-neutralizing antibodies (Eberhardt *et al.*, 2012). This observation suggests that vIL-10-based vaccines may not be able to interrupt an established persistent infection. Interestingly, development of antibodies against RhCMV vIL-10 in uninfected rhesus macaques immunized with plasmid vectors encoding for engineered, nonfunctional RhCMV vIL-10 variants resulted in reduction of RhCMV replication at the inoculation site and RhCMV shedding in bodily fluids during subcutaneous RhCMV challenge (Eberhardt *et al.*, 2013). Alternatively, for vIL-10s playing a significant role in virulence, deleted recombinant strains could be produced as attenuated vaccines as suggested for RhCMV (Chang & Barry, 2010).

The data presented in the previous section collectively indicate that vIL-10s, compared to cIL-10, have a restricted bioactivity profile favouring immunosuppressive activities. Based on this profile, several independent groups have suggested exploiting vIL-10s as potential immunosuppressive agents. Studies performed in laboratory animal models support this concept. Researchers have demonstrated the potential of some vIL-10s to induce localized immunosuppression in order to favour long-term engraftment of transplanted tissues (EBV

vIL-10) (Nast *et al.*, 1997; Qin *et al.*, 1996), reduce the host's foreign body reaction against implanted biomaterials (HCMV cmvIL-10) (van Putten *et al.*, 2009), or treat collagen-induced arthritis (EBV vIL-10) (Keravala *et al.*, 2006; Kim *et al.*, 2000; Lechman *et al.*, 1999; Ma *et al.*, 1998; Whalen *et al.*, 1999).

10. Concluding remarks

Most viruses have been co-evolving with their hosts for millions of years. During this process, viruses and hosts have been acting as strong sources of selection pressure on each other. Thus, viruses have been constantly selecting individuals among the host population that have the most efficient immune systems, while the continual improvement of the immune system has been selecting viruses that have evolved strategies to control the host immune response. Fundamental studies in immunology have demonstrated the key roles of cIL-10 in the immune system. The various independent acquisitions of IL-10 orthologues by viruses belonging to different viral genera, subfamilies and even families further support the importance of cIL-10 in the immune system. After their capture by the viral genome, cellular sequences evolve through positive selection to retain properties that are the most beneficial for the virus, and, sometimes, to acquire novel properties. The vIL-10s illustrate this concept. In comparison to their cellular orthologues, vIL-10s have evolved towards a more restricted bioactivity profile consisting mainly, but not exclusively, of immunosuppressive activities. Interestingly, studies on HCMV cmvIL-10 and LAcmvIL-10 demonstrate that evolution of a captured IL-10 gene in the viral genome has led to the expression of two different transcripts that have specific biological activities adapted to the replication and latent phases.

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Figure legends

Fig. 1. Schematic representation of the genomic intron/exon organization of human IL-10 (*H. sapiens*, Genbank ID: NP_000563) and vIL-10s encoded by the viruses listed in Table 1. Boxes and horizontal lines represent exons and introns, respectively. They are drawn to scale. The 5'- and 3'-UTRs of human IL-10 are not shown. The homology existing between each human IL-10 exons and virus IL-10s were investigated at the level of amino acid sequences using the accession numbers listed in Table 1 and the FASTA Sequence Comparison program (http://fasta.bioch.virginia.edu/fasta_www2/index.cgi) using default settings. Regions of vIL-10 DNA sequences encoding amino acid sequences homologous to human IL-10 protein domain encoded by each exon are drawn to scale using the following colour code: exon 1: red, exon 2: yellow, exon 3: blue, exon 4: green, exon 5: orange. Regions of vIL-10s for which no homology could be detected are presented in grey. HCMV cmvIL-10 and LAcmvIL-10 represent transcripts of the HCMV UL111A gene expressed during lytic and latent infections, respectively. The former retains the structure of the gene consisting of three exons and two introns, and the latter retains only the first intron, resulting in an in-frame stop codon 12 codons after the second exon.

Fig. 2. Maximum likelihood phylogenetic tree for cIL-10s, vIL-10s (listed in Table 1), the 134R protein encoded by Yaba-like disease virus, and selected members of the IL-20 family of cytokines. Sequences and methods used are described in supplementary file S1. The tree was build using MEGA (JTT+Γ substitution model) and 100 bootstrap replicates. Numbers of nodes indicate bootstrap confidence, where >70%. Cellular IL-10 and vIL-10s are collapsed into a single branch. Scale: substitutions per site.

672

673 **Fig. 3.** Bayesian consensus tree built using BEAST for cIL-10s and vIL-10s. Sequences and
674 methods used are described in supplementary file S1. Figures at nodes are posterior probabilities
675 (where >70%) of common ancestry. Branch lengths are arbitrary. Four positionally orthologous
676 sets of vIL-10 are framed. Independent gene acquisition events are marked by letters (A to I).

677

678 **Fig. 4.** Structure of cIL-10s and selected vIL-10s. a) Crystal structure of human IL-10 from the
679 IL-10/IL-10R1 complex (PDB ID: 1j7v (Josephson *et al.*, 2001)). IL-10 protomers are depicted
680 with helices rendered as cylinders. Helices are labelled. b) Ribbon diagram of the 1:2 IL-10/sIL-
681 10R1 complex viewed perpendicular to the twofold axis of IL-10 (reproduced with permission
682 from Josephson et al., 2001). c) to f) Superposition of host and viral IL-10s modeled using
683 human IL-10 as template (PDB ID: 1j7v). c) Human IL-10 (green, PDB ID: 1j7v (Josephson *et*
684 *al.*, 2001)) and EBV vIL-10 (blue, PDB ID: 1Y6M (Yoon *et al.*, 2005)). d) Human IL-10 (green)
685 and HCMV cmvIL-10 (brown, PDB ID: 1LQS (Jones *et al.*, 2002)). e) European eel IL-10 (red)
686 and AngHV1 vIL-10 (orange) (van Beurden *et al.*, 2011). f) Common carp IL-10 (green) and
687 CyHV-3 IL-10 (yellow) (van Beurden *et al.*, 2011). The interdomain angles of each IL-10
688 orthologue published previously are shown at the top of each complex.

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Table 1. Features of vIL-10s

Family Subfamily Genus	Virus name	Abbreviation	Locus	Accession number	Exon /Intron	Protein length (SP)	Main host species	Identity with host cIL-10	References
Herpesviridae									
Betaherpesvirinae									
Cytomegalovirus									
	Human cytomegalovirus	HCMV	UL111A/ cmvIL-10	AAR31656	3/2	176 (25)	Human	27.3%	(Kotenko *et al.*, 2000; Lockridge *et al.*, 2000)
			UL111A/ LA cmvIL-10	ACR49217	2/1	139 (24)		29.0%	(Jenkins *et al.*, 2004)
	Green monkey cytomegalovirus	GMCMV	UL111A (S)	AEV80459	4/3	185 (26)	Green monkey	28.2%	(Davison *et al.*, 2013)
	Rhesus cytomegalovirus	RhCMV	U111A	AAF59907	4/3	189 (31)	Macaque	25.0%	(Lockridge *et al.*, 2000)
	Baboon cytomegalovirus	BaCMV	vIL-10 (S)	AAF63436	4/3	191 (33)	Baboon	28.6%	(Lockridge *et al.*, 2000)
	Owl monkey cytomegalovirus	OMCMV	UL111A (S)	AEV80800	4/3	182 (21)	Owl monkey	30.3%	(Davison *et al.*, 2013)
	Squirrel monkey cytomegalovirus	SMCMV	UL111A (S)	AEV80955	4/3	178 (18)	Squirrel monkey	31.5%	(Davison *et al.*, 2013)
Gammaherpesvirinae									
Lymphocryptovirus									
	Epstein-Barr virus	EBV	BCRF1	CAD53385	1/0	170 (23)	Human	92.3%	(Arrand *et al.*, 1981)
	Bonobo herpesvirus	Bonobo-HV	LOC100970108 (S)	XP_003804206.1	1/0	169 (18)	Bonobo	94.3%	
	Rhesus lymphocryptovirus	RhLCV	BCRF1 (S)	AAK95412	1/0	177 (29)	Macaque	97.2%	(Franken *et al.*, 1996)
	Baboon lymphocryptovirus	BaLCV	vIL-10 (S)	AAF23949	1/0	171 (24)	Baboon	91.6%	
Macavirus									
	Ovine herpesvirus 2	OvHV-2	Ov2.5	AAX58040	5/4	182 (26)	Sheep	49.6%	(Meier-Trummer *et al.*, 2009)
Percavirus									
	Equid herpesvirus 2	EHV-2	ORF E7 (S)	AAC13857	1/0	179 (18)	Horse	90.4%	(Telford *et al.*, 1995)
Alloherpesviridae									
Cyprinivirus									
	Cyprinid herpesvirus 3	CyHV-3	ORF134	ABG42961	2/1	179 (17)	Common Carp	26.9%	(Aoki *et al.*, 2007)
	Anguillid herpesvirus 1	AngHV-1	ORF25	AFK25321	1/0	165 (19)	European eel	34.3%	(van Beurden *et al.*, 2010)

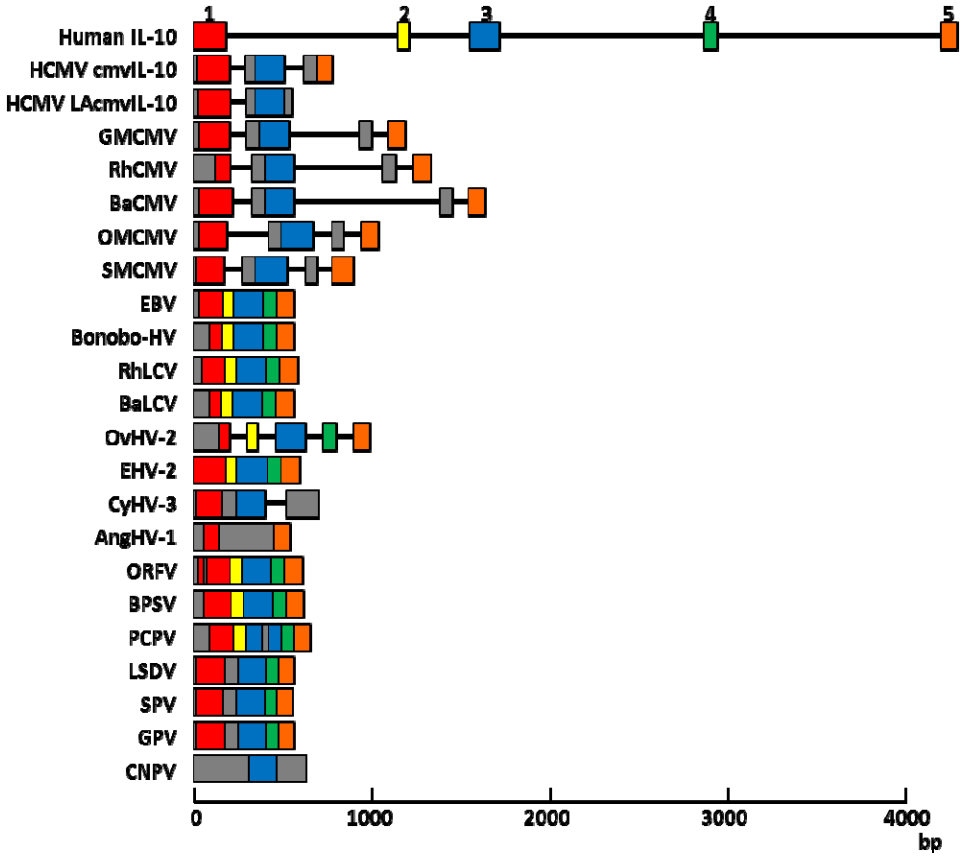
Table 1. cont.

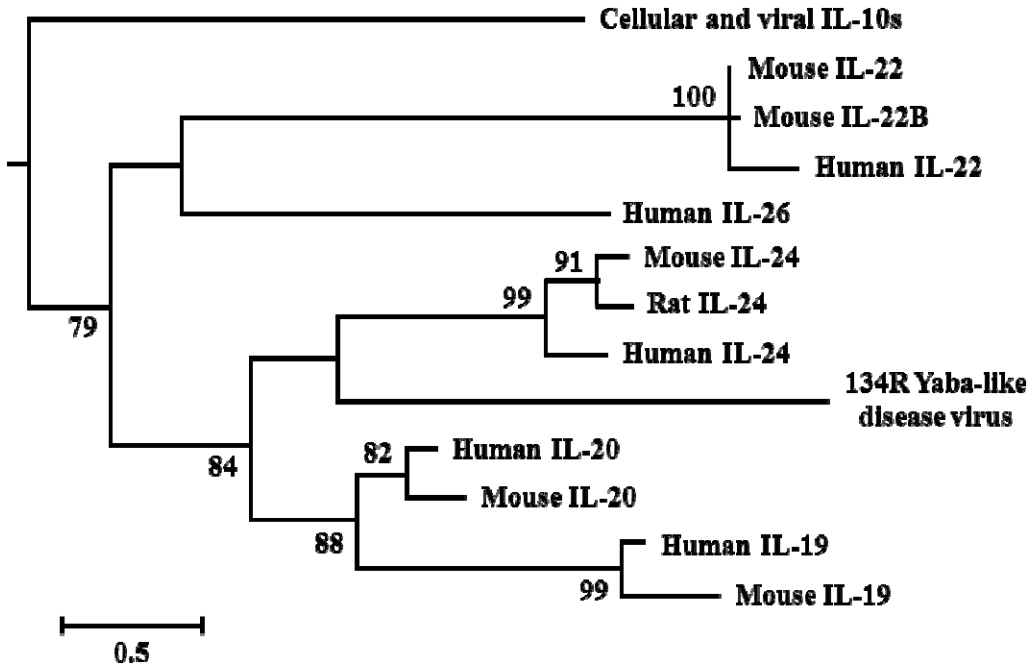
Family Subfamily Genus	Virus name	Abbreviation	Locus	Accession number	Exon /Intron	Protein length (SP)	Main host species	Identity with host cIL-10	References
Poxviridae									
Chordopoxivrinae									
Parapoxvirus									
	Orf virus	ORFV	ORF127	AAR98352	1/0	184 (22)	Sheep/ Goat	96.6% /97.3%	(Delhon *et al.*, 2004)
	Bovine papular stomatitis virus	BPSV	ORF127 (S)	AAR98483	1/0	185 (23)	Cattle	94.4%	(Delhon *et al.*, 2004)
	Pseudocowpox virus	PCPV	ORF127 (S)	ADC53770	1/0	199 (23)	Cattle	87.3%	(Hautaniemi *et al.*, 2010)
Capripoxvirus									
	Lumpy skin disease virus	LSDV	LSDV005 (S)	AAK84966	1/0	170 (23)	Cattle	45.7%	(Tulman *et al.*, 2001)
	Sheeppox virus	SPV	SPPV_03 (S)	NP_659579	1/0	168 (25)	Sheep	47.9%	(Tulman *et al.*, 2002)
	Goatpox virus	GPV	GTPV_gp003 (S)	YP_001293197	1/0	170 (27)	Goat	49.6%	(Tulman *et al.*, 2002)
Avipoxvirus									
	Canarypox virus	CNPV	CNPV018 (S)	NP_955041	1/0	191 (20)	Passeriform birds	*	(Tulman *et al.*, 2004)

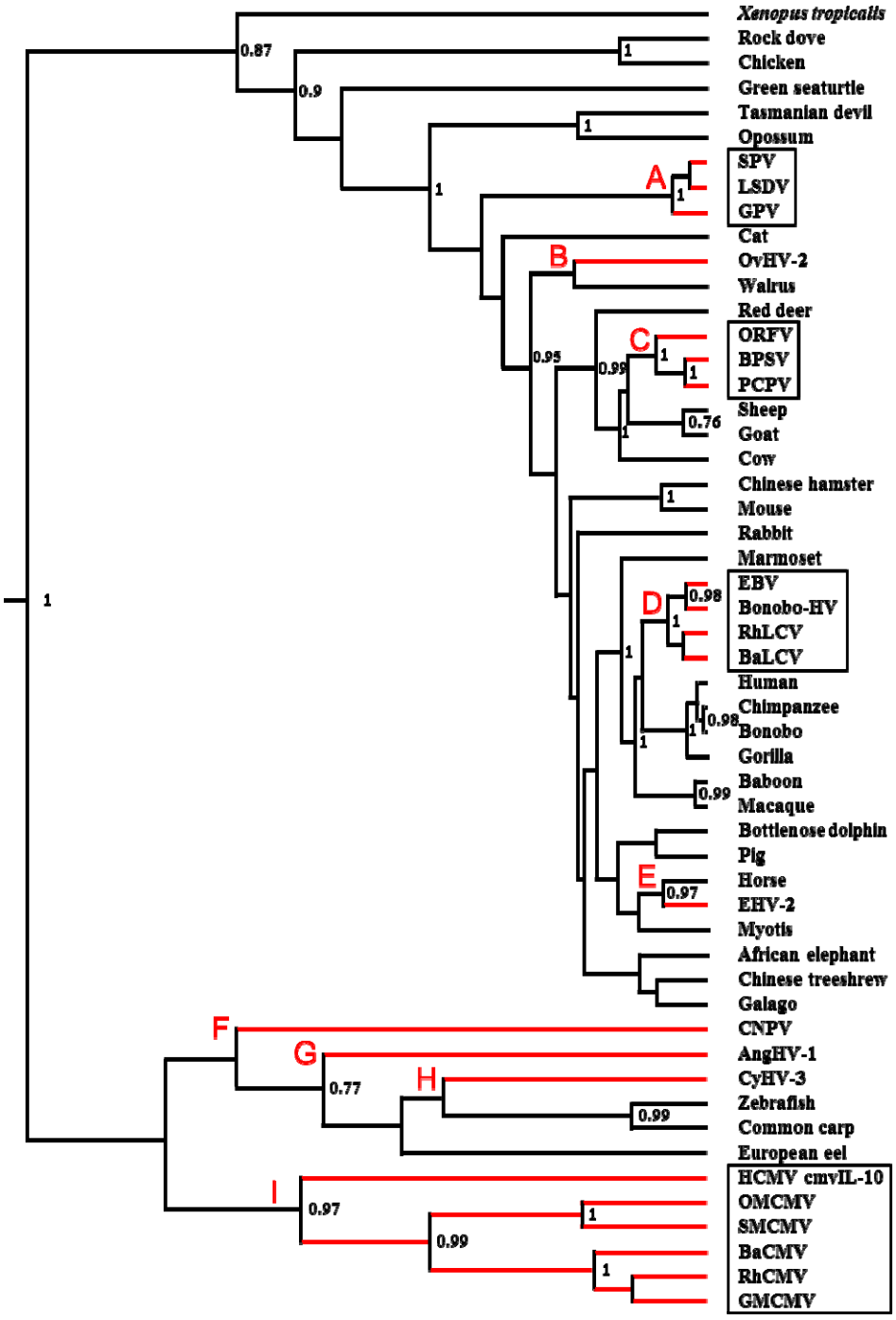
Tables

Table 1. Features of vIL-10s.

Exon number and protein length were determined based on the sequences available in the public databases. Signal peptides were predicted by using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). Mature proteins (excluding signal peptide sequences) were compared using the FASTA sequence comparison program (http://fasta.bioch.virginia.edu/fasta_www2/). Protein sequence accession numbers for the hosts are as follows: *Homo sapiens* (human; NP_000563), *Macaca mulatta* (rhesus macaque; NP_001038192), *Papio anubis* (baboon; XP_003893246), *Pan paniscus* (bonobo; XP_003822966.1), *Ovis aries* (sheep; emb|CAG38358), *Capra hircus* (goat; ABI20513), *Bos taurus* (cow; NP_776513), *Equus caballus* (horse; NP_001075959), *Cyprinus carpio* (common carp; BAC76885), *Anguilla anguilla* (European eel; AEL99923). SP: signal peptide; * no IL-10 consensus sequence is available for passeriform birds, S: Viruses for which the only available data is the vIL-10 sequence.


1
2
3
4
5
Human IL-10
HCMV cmvIL-10
HCMV LAcmvIL-10
GMCMV
RhCMV
BaCMV
OMCMV
SMCMV
EBV
Bonobo-HV
RhLCV
BaLCV
OvHV-2
EHV-2
CyHV-3
AngHV-1
ORFV
BPSV
PCPV
LSDV
SPV
GPV
CNPV
0
1000
2000
3000
4000
bp


Cellular and viral IL-10s
Mouse IL-22
100
Mouse IL-22B
Human IL-22
Human IL-26
91
Mouse IL-24
99
Rat IL-24
79
Human IL-24
134R Yaba-like
disease virus
82
Human IL-20
84
Mouse IL-20
88
Human IL-19
99
Mouse IL-19
0.5


Xenopus tropicalis
Rock dove
Chicken
Green seaturtle
Tasmanian devil
Opossum
A
SPV
LSDV
GPV
Cat
B
OvHV-2
Walrus
Red deer
C
ORFV
BPSV
PCPV
Sheep
Goat
Cow
Chinese hamster
Mouse
Rabbit
Marmoset
D
EBV
Bonobo-HV
RhLCV
BaLCV
Human
Chimpanzee
Bonobo
Gorilla
Baboon
Macaque
Bottlenose dolphin
Pig
E
Horse
EHV-2
Myotis
African elephant
Chinese treeshrew
Galago
F
CNPV
G
AngHV-1
H
CyHV-3
Zebrafish
Common carp
European eel
I
HCMV cmvIL-10
OMCMV
SMCMV
BaCMV
RhCMV
GMCMV
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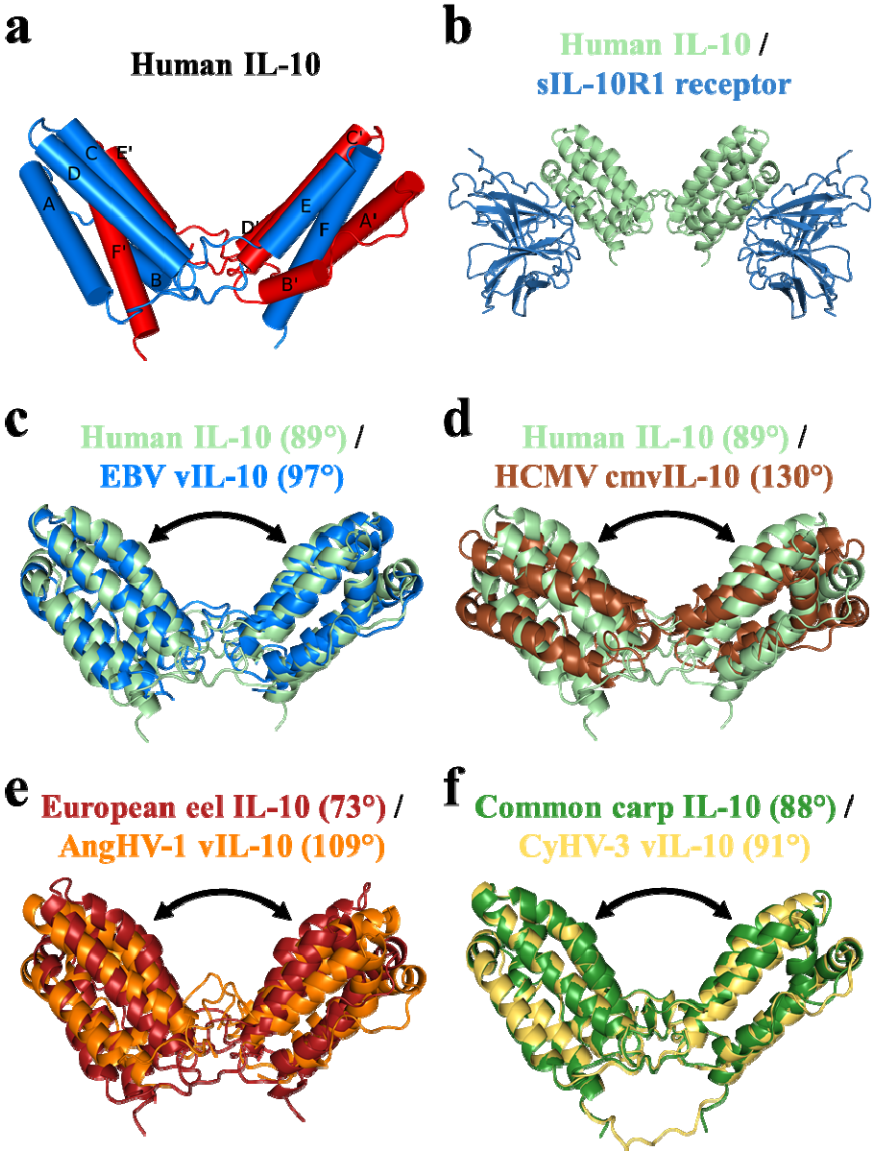
0.9
1
0.95
0.99
0.76
0.98
0.97
0.77
0.99
0.97


a
Human IL-10
C
E'
D
A
F'
B
C'
D'
E
F
A'
B'
b
Human IL-10 /
sIL-10R1 receptor
c
Human IL-10 (89°) /
EBV vIL-10 (97°)
d
Human IL-10 (89°) /
HCMV cmvIL-10 (130°)
e
European eel IL-10 (73°) /
AngHV-1 vIL-10 (109°)
f
Common carp IL-10 (88°) /
CyHV-3 vIL-10 (91°)