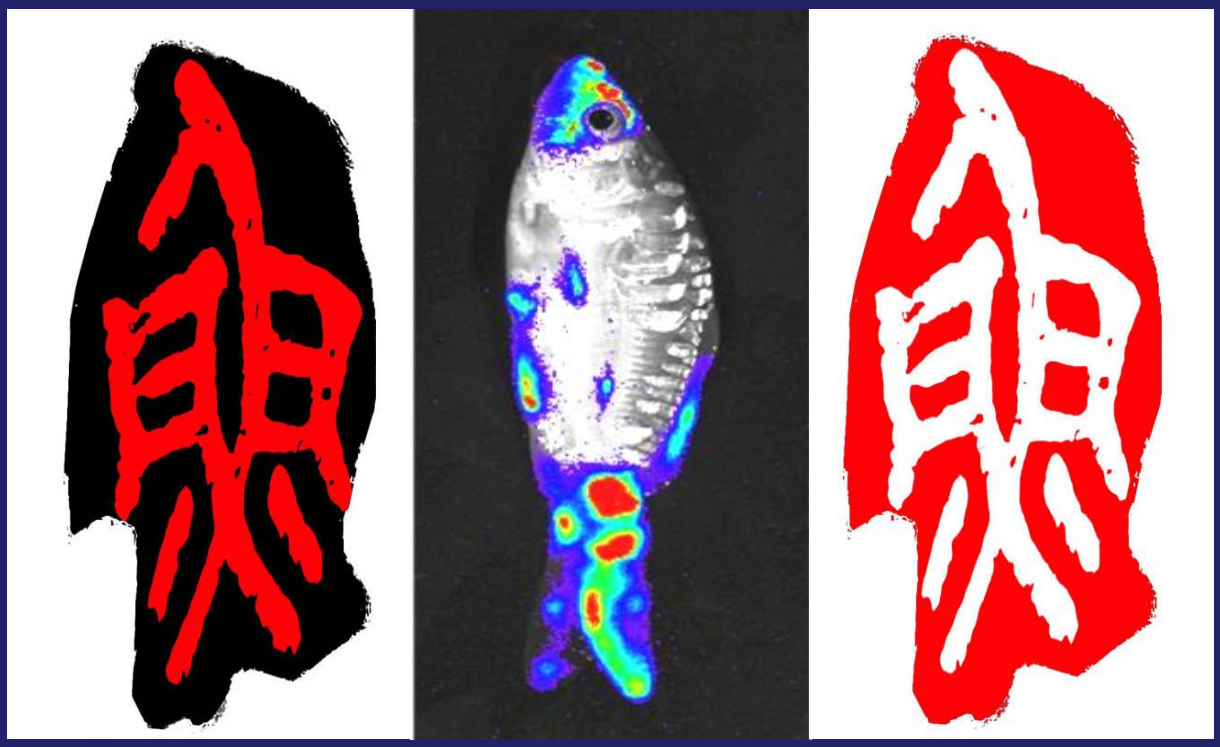
Académie Universitaire Wallonie - Europe
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Service d'Immunologie et de Vaccinologie

Study of the roles of Cyprinid herpesvirus 3 ORF134 in the biology of the infection

Etude des rôles du gène ORF134 dans la biologie de l'infection de l'Herpèsvirus cyprin 3



Ping OUYANG

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences Vétérinaires

Année académique 2013-2014

Cover page : the central picture represents the analysis by *in vivo imaging system* of a carp infected with a Cyprinid herpesvirus 3 recombinant strain expressing firefly luciferase as a reporter gene. The flanking pictures represent the word “fish” in old Chinese.

Académie Universitaire Wallonie - Europe
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Service d'Immunologie et de Vaccinologie

Study of the roles of Cyprinid herpesvirus 3 ORF134 in the biology of the infection

Etude des rôles du gène ORF134 dans la biologie de l'infection de l'Herpèsvirus cyprin 3

Promotor : Prof. Alain Vanderplasschen

Ping OUYANG

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences Vétérinaires

Année académique 2013-2014

« *The way ahead is long and has no ending, yet high and low I'll search with my will unbending.* »
« *路漫漫其修远兮, 吾将上下而求索.* »

屈原 (Qū Yuán, 343–278 BCE)

Acknowledgments

The work presented in this thesis has been carried out in the laboratory of Immunology-Vaccinology, faculty of Veterinary Medicine, University of Liège, Belgium. I am extremely grateful to my promotor **Prof. Alain Vanderplasschen** for giving me the opportunity to study in his lab. His trust, his permanent support and his passion for science have been an essential source of motivation and inspiration for me. I will always admire him for his constant positive attitude when facing problems and for his trust that the "good must win".

I would like to express my gratitude to the members of my PhD committee who monitored my work progress and took effort in reviewing and providing me with insightful comments: **Prof. Daniel Desmecht** and **Dr. François Lieffrig**.

The present work was at the origin of several fruitful collaborations with other laboratories. I would like to thank **Prof. Ruddy Wattiez** and **Dr Baptiste Leroy** (Proteomic and Microbiology, University of Mons, Belgium) for their interest in our project and their willingness to perform proteomic analyses. Many thanks to **Dr. Andrew Davison** and **Dr. Derek Gatherer** (Centre for Virus Research, University of Glasgow, United Kingdom) for their contribution on the study of viral IL-10 evolution. Many thanks also go to **Dr. Adrie Westphal** (Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands) for his contribution on viral IL-10 structural analyses.

The four years spent in the laboratory of Immunology-Vaccinology have not only been my source of scientific experiences, but also of invaluable human relationships. Many thanks go to all the members of the Immunology-Vaccinology lab. I would like to start with the members of the fish group. This very rich environment is composed of 7 persons originating from 6 different countries. **Dr. Krzysztof Rakus** and his wife **Dr. Joanna Jazowiecka-Rakus** are from Poland, thanks for their endless support and invaluable suggestions on fish immunology. **Dr. Anca Reschner** comes from Romania, thanks for her constant help and nice discussions. **Maygane Ronsmans** is a beautiful Belgian girl, thanks for her help during the doctoral formation courses. **Maxime Boutier** is a very nice French guy and it has been a great pleasure to work with him. **Dr. Ma. Michelle Penaranda**, the new addition in the lab, comes from the Philippines. Many thanks to these people for their constant help and the very friendly atmosphere they are creating in the fish group office.

I also would like to thank members of the fish group who left the lab. **Dr. Guillaume Fournier**, his precious help has been essential to the progress of my thesis. Thanks to **Dr. Robert Vrancken**, **Dr. Stalin Raj**, **Dr. Benjamin Michel**, **Dr. Hélène Schroeder** and **Dr. Bérénice Costes** for helping me settle in when I arrived in Belgium.

The laboratory of Immunology-Vaccinology is composed of three sub-groups that are constantly interacting. I would like to thank the people from the two other sub-groups I had the chance to work with. Thanks to the people of the group lead by **Prof. Laurent Gillet**: **Dr Bénédicte Machiels, Dr Céline Lété, Dr Sylvie François, Dr Sarah Vidick, Bérengère Boutard, Bilal Latif and Mickael Dourcy,** as well as the group what lead by **Dr Benjamin Dewals**: **Dr Leonor Plameira, Dr Steven van Beurden, Françoise Myster, Océane Sorel.**

The three sub-groups of the Immunology-Vaccinology lab benefit from the work of dedicated technicians and secretaries. I'm thankful to **Cédric Delforge, Emeline Deglaire, Christine Thys, Jérémy Dumoulin, Nathalie Poncelet, Antoine Guillaume, François Massart, Dominique Ziant and Charles Gaspar** who contributed directly or indirectly to experiments. Last but not the least, I would like thank our secretaries **Christina Espert** and **Lorère Dams**, who made my life more convenient in the lab.

I would like to thank my master supervisor **Prof. Liancheng Lei** (College of Animal Husbandry and Veterinary Medicine, Jilin University, China) for his permanent encouragement and constant help. Without him, I would not have the chance to study abroad for 4 years.

I also would like to acknowledge my Chinese friends in Belgium, **Xuerong Jiang**, **Huijun Cheng**, **Zhiyan Zhang**, **Wanbo Li**, **Qiongzhong Chen**, **Yongzhen Li**, **Ji Liu**, **Ming Fang**, **Xuewen Xu**, **Cheng Liu, Xin Zhang,** who offered me their help to solve the numerous practical problems that I had to face when arriving in Belgium. They made also my life in Belgium more colorful.

Many thanks go to my beloved family for their loving consideration and their great confidence in me throughout all these years. Most importantly, I would like to thank my parents for supporting me spiritually throughout my life. I must acknowledge my boyfriend, **Dr. Lizi YIN**, without his love, his permanent encouragement and constant help, I would not have finished my thesis and life in Belgium would not have been so nice.

Being in Belgium to complete a PhD thesis is definitely one of the most interesting, stimulating and exciting experiences of my live. This experience has been possible thanks to the financial support of the **Chinese Scholarship Council** (Application No.2009617025).

Liège, 15th September 2013

Ping Ouyang

List of abbreviations

2D-LC MS/MS:	Two-dimensional liquid chromatography tandem mass spectrometry
α gene:	Immediate early gene
aa:	Amino acid
ACHV:	Atlantic cod herpesvirus
AciHV-1:	Acipenserid herpesvirus 1
AciHV-2:	Acipenserid herpesvirus 2
AlHV-1:	Alcelaphine herpesvirus 1
AngHV-1:	Anguillid herpesvirus 1
APCs:	Antigen presenting cells
AtHV-3:	Ateline herpesvirus 3
Au:	Goldfish fin cell
β gene :	Early gene
BAC:	Bacterial artificial chromosome
BaCMV:	Baboon cytomegalovirus
BaLCV:	Baboon lymphocryptovirus
BHV-4:	Bovin herpesvirus 4
BMDCs:	Bone marrow-derived dendritic cells
BoHV-1:	Bovine herpesvirus 1
BoHV-5:	Bovine herpesvirus 4
BoHV-5:	Bovine herpesvirus 5
Bonobo-HV:	Bonobo herpesvirus
bp:	Base pair
BPSV:	Bovine papular stomatitis virus
CaF-2:	Carp fin cell
CCB:	*Cyprinus carpio* brain cell
CCG:	*Cyprinus carpio* gill cell
CCMV:	Chimpanzee cytomegalovirus
CCV:	Channel catfish virus (= IcHV-1)
CD:	Cluster of differentiation
cDNA:	Complementary DNA
CeHV-2:	Cercopithecine herpesvirus 2
CeHV-9:	Cercopithecine herpesvirus 9
CHV:	Carp herpesvirus (= CyHV-1)
CHX:	Cycloheximide
cIL-10:	Cellular Interleukin-10
CMV:	Cytomegalovirus
CNGV:	Carp nephritis and gill necrosis virus
CNPV:	Canarypox virus
CPE:	Cytopathic effect

CRF2:	Class II cytokine receptor family
CSIF:	Cytokine synthesis inhibitory factor
Ct:	Threshold cycle
CXCL10:	CXC chemokine ligand 10
CyHV-1:	Cyprinid herpesvirus 1(= CHV)
CyHV-2:	Cyprinid herpesvirus 2
CyHV-3:	Cyprinid herpesvirus 3
DC:	Dendritic cells
DC-SIGN:	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
Del :	Deleted
DMEM:	Dulbecco's modified essential medium
DNA:	Deoxyribonucleic acid
dUTPase:	Deoxyuridine triphosphate pyrophosphatase
E:	Early
EBV:	Epstein-Barr virus (= HHV-4)
EEDV:	Epizootic epitheliotropic disease virus (= SalHV-3)
EGFP:	Enhanced green fluorescent protein
EHV-1:	Quid herpesvirus 1
EHV-2:	Quid herpesvirus 2
EHV-4:	Quid herpesvirus 4
ER:	External repeats
FBR:	Foreign body reaction
FCS:	Fetal calf serum
FHM:	Fathead minnow cell
FV-4:	Frog virus 4 (= RaHV-2)
*gal*K:	Galactokinase
GaHV-1:	Gallid herpesvirus 1
GaHV-2:	Gallid herpesvirus 2
GaHV-3:	Gallid herpesvirus 3
γ gene :	Late gene
G-CSF:	Granulocyte colony-stimulating factor
GFHNV:	Goldfish hematopoietic necrosis virus
GMCMV:	Green monkey cytomegalovirus
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
gp:	Glycoprotein
GPCR:	G-protein couple receptor
GPV :	Goatpox virus
HCMV:	Human cytomegalovirus (= HHV-5)
HHV-1:	Human herpesvirus 1
HHV-2:	Human herpesvirus 2
HHV-3:	Human herpesvirus 3

HHV-4:	Human herpesvirus 4
HHV-5:	Human herpesvirus 5
HHV-6:	Human herpesvirus 6
HHV-7:	Human herpesvirus 7
HHV-8:	Human herpesvirus 8
HPV:	Herpesvirus salmonis
HSV-1:	Herpes simplex type 1
HVA:	Herpesvirus anguillae (= AngHV-1)
IcHV-1:	Ictalurid herpesvirus 1
IcHV-2:	Ictalurid herpesvirus 2
IcmHV:	Ictalurus melas *herpesvirus* (= IcHV-1)
IE:	Immediate early
IFN:	Interferon
IgA, G, M:	Immunoglobulin A, G, M
IL:	Interleukin
IL-10R:	IL-10 receptor
iNOS:	Inducible nitric oxide synthase
IPNV:	Infectious pancreatic necrosis virus
IR:	Internal repeats
IVIS:	*in vivo* bioluminescence imaging system
Jak1:	Janus kinase 1
KF-1:	Koi fin cell
KFC:	Koi fin cell
KHV:	Koi herpesvirus
KHVD:	Koi herpesvirus disease
L:	Late
LAcmvIL-10:	Latency associated cytomegalovirus IL-10
LPS:	Lipopolysaccharides
LSDV:	Lumpy skin disease virus
LTHV:	Lucké tumor herpesvirus (= RaHV-1)
LUC:	Luciferase
LTR:	Left terminal repeats
McHV-1:	Macacine herpesvirus 1
McHV-4:	Macacine herpesvirus 4
McHV-8:	Macacine herpesvirus 8
MDDCs:	Monocyte-derived dendritic cells
MeHV-1:	Meleagrid herpesvirus 1
MEM:	Minimum essential medium
MHC class II B:	Major Histocompatibility Complex class II B
MHC:	Major histocompatibility complex
MOI:	Multiplicity of infection

mRNA:	Messenger RNA
MS:	Mass spectrometry
MuHV-1:	Murid herpesvirus 1
MuHV-2:	Murid herpesvirus 2
MuHV-4:	Murid herpesvirus 4
NGF-2:	Epithelial-like cell line from fins of coloured carp 2
NGF-3:	Epithelial-like cell line from fins of coloured carp 3
NK cells:	Natural killer cells
OMCMV:	Owl monkey cytomegalovirus
OMV:	Oncorhynchus masou virus
ORF:	Open reading frame
ORFV:	Orf virus
OsHV-1:	Ostreid herpesvirus 1
OvHV-2:	Ovine herpesvirus 2
PAA:	Phosphonoacetic acid
PaHV-1:	Panine herpesvirus 1
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline
PCPV:	Pseudocowpox virus
PDCs:	Plasmacytoid dendritic cells
PeHV-1:	Percid herpesvirus 1
p.f.u.:	Plaque forming unit
PGE2:	Prostaglandin E2
PHA:	Phytohaemagglutinin
PrV:	Pseudorabies virus
PsHV-1:	Psittacid herpesvirus 1
RaHV-1:	Ranid herpesvirus 1
RaHV-2:	Ranid herpesvirus 2
RELP:	Restriction fragment length polymorphism
RhCMV:	Rhesus cytomegalovirus
RhLCV:	Rhesus lymphocryptovirus
RT-PCR:	Reverse transcription PCR
RT-qPCR:	Real-time quantitative PCR
RTR:	Right terminal repeats
SaHV-2:	Saimiriine herpesvirus 2
SalHV-1:	Salmonid herpesvirus 1
SalHV-2:	Salmonid herpesvirus 2
SalHV-3:	Salmonid herpesvirus 3
SD:	Standard deviation
SHV:	Steelhead herpesvirus
sIL10 R1:	Soluble IL10 receptor 1

SMCMV:	Squirrel monkey cytomegalovirus
SPV:	Sheeppox virus
STAT:	Signal transduction and transcription
SuHV-1:	Suid herpesvirus 1
SVC:	Spring viraemia of carp
Th1:	T helper 1
TK:	Thymidine kinase
TLEV:	Tilapia larvae encephalitis virus
TNFR:	Tumor necrosis factor receptor
Tol/FL:	Silver carp fin cell
TuHV-1:	Tupaiid herpesvirus 1
Tyk2:	Tyrosine kinase 2
vIL-10:	Viral IL-10
VNTR:	Variable number of tandem repeats

Preamble ……1

Introduction …….. 3

1st chapter: 5
"The order *Herpesvirales*"

1. Introduction 6
2. Virus structure 6
3. Genomic features 7
4. Common biological properties 7
5. Biological cycle 7
   5.1 Lytic infection 7
   5.2 Latent infection 9
6. Classification of the order *Herpesvirales* 9
   6.1 The *Herpesviridae* family 10
   6.2 The *Malacoherpesviridae* family 10
   6.3 The *Alloherpesviridae* family 11
7. References 12

2nd chapter: 16
"Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research"

Abstract 17
1. Introduction 18
2. Characterization of CyHV-3 18
   2.1 General description 18
   2.2 *In vitro* replication 21
   2.3 Temperature restriction 21
   2.4 Geographical distribution 22
   2.5 Presence of CyHV-3 in natural environment 22
3. Disease 23
   3.1 Disease characteristics 23
   3.2 Host range and susceptibility 24
   3.3 Pathogenesis 24
   3.4 Transmission 26
   3.5 Diagnosis 26

3.6 Vaccination 27
4. Host-pathogen interactions 27
4.1 Genetic resistance of carp strains to CyHV-3 27
4.2 Immune response of carp against CyHV-3 28
5. Conclusion 30
6. Competing interests 31
7. Author's contributions 31
8. Acknowledgments 31
9. References 32

3rd chapter: 40
"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"

Abstract 41
1. Introduction 42
2. Discovery of vIL-10s 42
3. Genetic structure of IL-10 homologues 43
4. Origin and evolution of vIL-10s 43
5. Protein structure of IL-10 homologues 45
6. Transcriptomic and proteomic expression of vIL-10 genes 46
7. Ligand-receptor complexes formed by IL-10 homologues 47
8. Biological activities of IL-10 homologues 48
8.1 Biological activities of cIL-10 48
8.2 Biological activities of vIL-10s 49
9. Viral IL-10s as a topic of applied research 56
10. Concluding remarks 57
11. Acknowledgements 57
12. References 58
Supplementary material S1 70
Supplementary material S2 71

Objectives …….. 72

Experimental section …… 74

1st chapter: 75

"The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*"

2nd chapter: 95

"Development of a Safe and Efficacious Attenuated Recombinant Vaccine against Cyprinid herpesvirus 3"

Discussion and perspectives …… 112

Summary……..……... 119

References ……... 122

Preamble

Preamble

Common carp (*Cyprinus carpio*) is cultivated for human consumption worldwide. It is one of the most important freshwater species in aquaculture with a world production of 3.4 million metric tons per year (estimation from the FAO for 2010). While the common carp represents a cheap source of animal proteins, its coloured subspecies koi (*Cyprinus carpio koi*) is cultivated as an expensive pet fish for personal pleasure or worldwide competitive exhibitions. The price of individual collectable subjects fluctuates between 1 to 30 K euros, but can reach much higher prices. In the late 1990s, a highly contagious and virulent disease began to cause severe economic losses in these two carps industries worldwide. The causative agent of the disease was initially called koi herpesvirus (KHV). It has been renamed as cyprinid herpesvirus 3 (CyHV-3) in 2005 and classified in the *Alloherpesviridae* family of the *Herpesvirales* order.

In addition to its economic importance, CyHV-3 has several qualities as a fundamental model of infection: (i) It is phylogenically distant from the vast majority of herpesviruses that have been studied so far. (ii) It can be studied in laboratories by infection of its natural host (homologous virus-host model). (iii) The sequence of its genome published in 2007 revealed a fascinating virus with unique properties in the *Herpesvirales*, such as an extremely large genome (295 Kb), a high number of genes which are not homologous to known viral sequences, and genes that are normally found exclusively in the *Poxviridae*. (iv) Interestingly, the sequencing of the CyHV-3 genome revealed several genes potentially encoding proteins involved in immune evasion mechanisms. Among these genes, ORF134 encodes a homologue of carp Interleukin 10 (IL-10). The identification of this ORF in the CyHV-3 genome represents an opportunity to study *in vitro* and *in vivo* the roles of a viral IL-10 (vIL-10) homologue using a homologue virus-host model. The present thesis was devoted to the study of this viral gene.

The structure of this manuscript is as follows. It starts with an introduction on the *Herpesvirales* order, on CyHV-3 and on the viral Interleukin-10 homologues encoded by viruses. While the first section is brief and general, the second and the third represent review manuscripts that have been published (in *Veterinary research*) or accepted for publication (in *Journal of General Virology*), respectively. After the introduction, the objectives of the thesis are briefly exposed. The results obtained during this thesis are then presented in two chapters. The first chapter has been published in *Veterinary Research*, while a manuscript including the results of the second chapter is under preparation for publication. In the last section of this manuscript, the main results are discussed and potential perspectives are presented.

Introduction

Preamble

The introduction of this thesis is structured in three chapters. The first chapter presents a brief description of the order *Herpesvirales.* The general properties of the viruses belonging to this order and the families it contains are described. This section of the manuscript is an update and adapted version of a text used in most theses of the host lab on herpesviruses. The second chapter of this introduction summarized the knowledge on Cyprinid herpesvirus 3 (CyHV-3) available in the literature when this thesis was printed. It represents a review has been published in Veterinary research. Ping Ouyang is second author of this review. The third and last chapter of the introduction represents a review on viral homologues of Interleukin-10 encoded by viruses (vIL-10). Ping Ouyang is co-first author of this manuscript.

Introduction

1st chapter:

The order *Herpesvirales*

1. Introduction

At the border of living and non-living, viruses are submicroscopic biological agents consisting of nucleic acid and protein shell which may be multilayered. They cannot replicate in the extracellular medium and reproduce as obligate intracellular parasites in the host organism. Since the description of the tobacco mosaic virus at the end of the 19th century, thousands of viruses were described in every ecosystem. They infect bacteria, plants and animals (Dimmock *et al.*, 2007). The International Committee on Taxonomy of viruses (ICTV) developed universal systems for classifying viruses. In the current ICTV taxonomy, six orders have been established, the *Caudovirales*, the *Herpesvirales*, the *Mononegavirales*, the *Nidovirales*, the *Picornavirales* and the *Tymovirales* (King *et al.*, 2012).

Members of the order *Herpesvirales* are enveloped viruses with a linear double-stranded DNA (dsDNA) genome. They share an identical structure. A densely packed DNA core is contained in an icosahedral capsid. The capsid is embedded in a complex proteinaceous layer called the tegument. A lipid envelope containing numerous viral glycoproteins forms the outermost structure of the viral particle (McGeoch *et al.*, 2008). Most of the members of the order *Herpesvirales* have been shown to realize two distinct phases in their life cycle: lytic replication characterized by a transcription program where immediate-early (IE), early (E), and late (L) genes are expressed successively; and latency, consisting of the maintenance of the viral genome as a non-integrated episome and the expression of a limited number of viral genes and microRNAs (Roizman & Pellet, 2007). Upon reactivation, latency reverses to a lytic replication.

The origin of the order *Herpesvirales* has been estimated at several hundred million years ago (Davison, 2002). So far, approximately 135 members have been isolated from oyster, fish, amphibian, reptile, bird and mammal species, including human (Davison *et al.*, 2009). Herpesviruses have mainly co-evolved with their host and in most cases are well adapted to them. This adaption is demonstrate that the ability of most herpesviruses to persist in the host species without inducing lethal infection.

The order *Herpesvirales* contains three families, the *Herpesviridae* (comprising viruses infecting mammals, birds and reptiles), the *Alloherpesviridae* (comprising viruses infecting fish and amphibians) and the *Malacoherpesviridae* (comprising viruses infecting mollusks) families. Below, we will first provide a general and brief description of the structure, the genome, the common biological properties and the replication cycle of the members of the order *Herpesvirales*. Next, we will discribe briefly the biological specificities of the three families.

2. Virus structure

Every virus classified in the order *Herpesvirales* possesses an identical structure (Ackermann, 2004). Their genome is protected by an icosahedral capsid with diameter of approximately 100 nm. The capsid is composed of 162 capsomers (150 hexons and 12 pentons) (Figure 1). This nucleocapsid is surrounded by an amorphous layer of proteins termed tegument, which contains proteins mainly

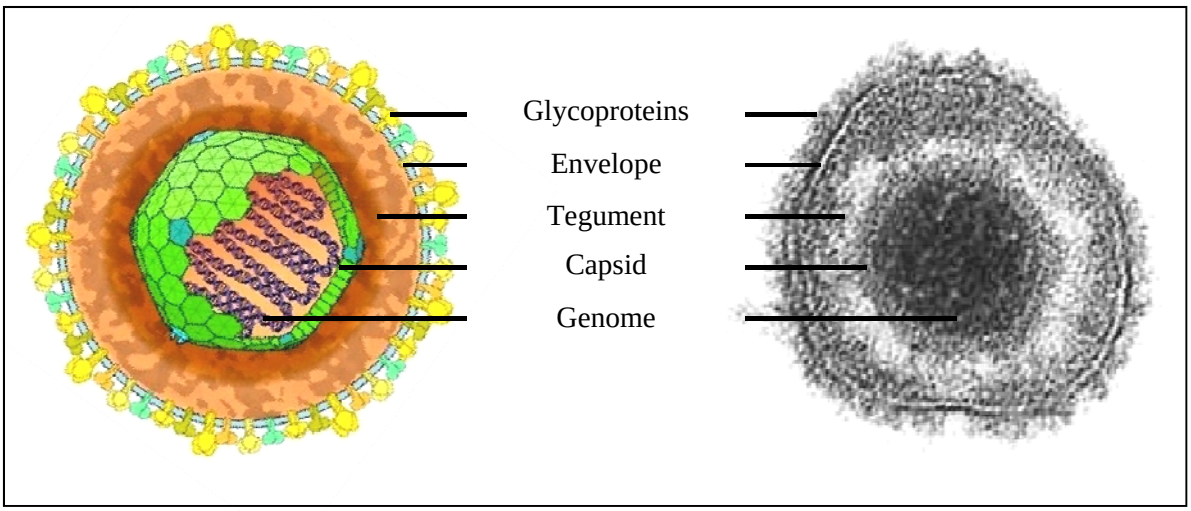


Figure 1. Herpesvirus structure. Schematic representation and electron microscopy picture of a viral particle.

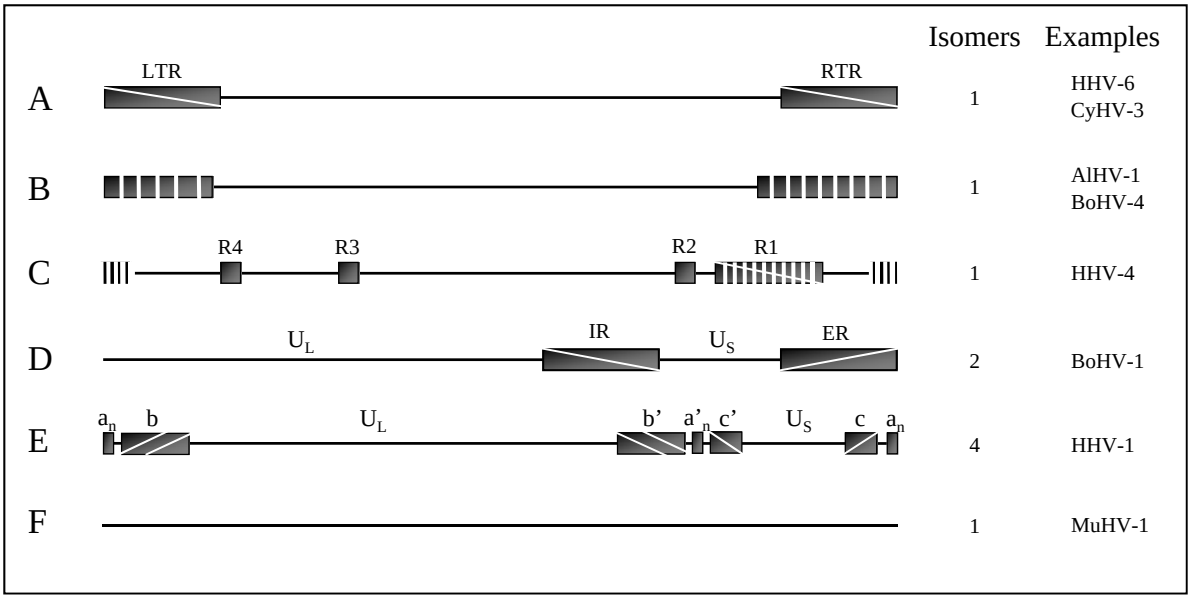


Figure 2. The order *Herpesvirales* regroups 6 classes of genome. Horizontal lines represent unique regions. Rectangles represent left and right terminal repeats (LTR and RTR, respectively) for A group; internal repeats R1 to R4 of the C group and internal and external repeats (IR and ER) for the D group. Terminal repeats of the E group are constituted by two parts. One is composed by *n* copies of the *a* sequence near the larger *b* sequence. The other one is composed by the repeated *a* sequences followed by a *c* sequence. Terminal sequences a_nb and ca_n are inversed and are separated by long (U_L) and short (U_S) unique sequences. In the B group, terminal sequences are repeated a variable number of times at each extremity. In the D group, U_S can be inverted compared to the U_L giving two different isomers. In the E group, U_L and U_S regions can also be inverted generating four different isomers. Terminal repeats were not described in the F group. *Human herpesvirus 1* (HHV-1), *4* (HHV-4) and *6* (HHV-6), *Alcelaphin herpesvirus 1* (AlHV-1), *Bovin herpesvirus 1* (BoHV-1) and *4* (BoHV-4), *Murin herpesvirus 1* (MuHV-1) and *Cyprinid herpesvirus 3* (CyHV-3) were chosen as examples (adapted from Roizman *et al.*, 2007).

involved in gene expression regulation. Finally, a lipid envelope bearing viral glycoproteins is covering the elements listed above to form a spherical particle of approximately 150 to 300 nm in diameter (Figure 1).

3. Genomic features

Herpesvirus genome is a long dsDNA molecule, linear in the capsid, but circular once it penetrates the nucleus of the host cell (Roizman & Pellet, 2007). Depending of the virus species, the guanine plus cytosine (G+C) percentage varies from 31 to 75% while the genome length varies from 120 to 295 kilo base pairs (kbp) (Aoki *et al.*, 2007; Roizman & Pellet, 2007). The genome contains variable internal and terminal repeated sequences. Based on the arrangement of these sequences, herpesvirus genomes have been classified in 6 different groups (Figure 2) (Roizman & Pellet, 2007). All herpesvirus genomes contain at their termini conserved signals for packaging of the DNA into capsids (Roizman & Pellet, 2007).

4. Common biological properties

Herpesviruses seem to share 4 important biological properties (Ackermann, 2004). Firstly, they encode their own enzymes for nucleic acid synthesis. Secondly, both viral DNA replication and assembly of the nucleocapsid take place in the nucleus of the infected cell. Thirdly, production of progeny viral particles leads to the lysis of the infected cell. Finally, even if this not firmly demonstrated for the *Alloherpesviridae* and *Malacoherpesviridae* families, all studied herpesviruses are able to establish a latent infection in their natural host.

5. Biological cycle

Herpesviruses have two distinct phases in their life cycle: lytic and latent infection. The characterization of these two phases is based on the study of members of the *Herpesviridae* family.

5.1 Lytic infection

The herpesvirus multiplication cycle is illustrated in Figure 3. It starts with the virion attachment on the host cell surface mediated by the interaction of viral glycoproteins with their cellular receptors. For example, human herpesvirus 1 (HHV-1) first binds to the cells through interaction of glycoproteins gC and gB with some cellular proteoglycans such as heparan sulfate (Spear, 2004). A stronger attachment is then mediated by the interaction of gD to its specific cellular receptor (Spear, 2004).

After fusion of the viral envelope with the plasma membrane (or eventually endocytic vesicles), the nucleocapsid and tegument proteins are delivered in the cytoplasm where microtubules bring the nucleocapsid surrounded by the tegument close to the nucleus (Figure 3) (Sodeik *et al.*,

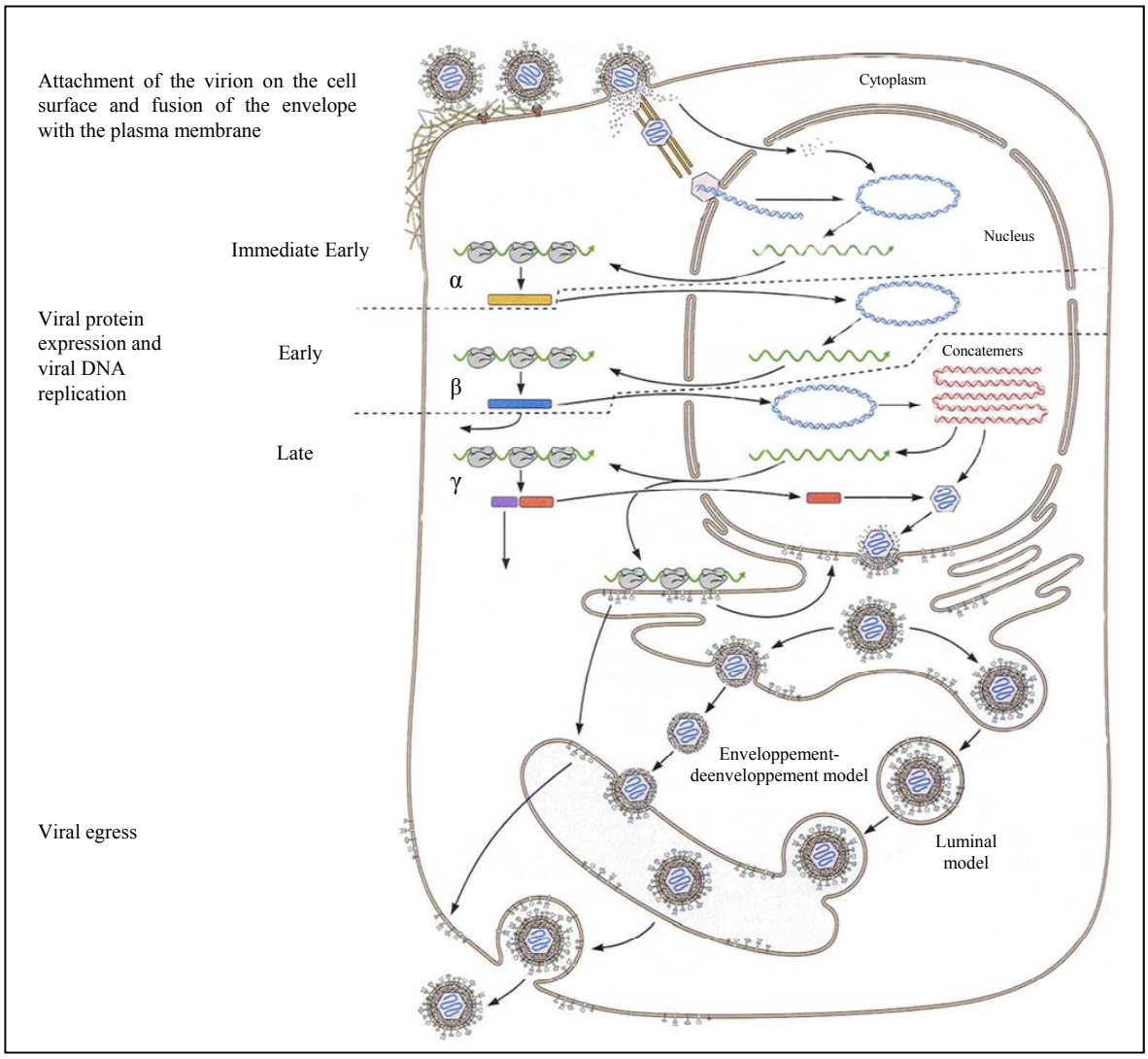


Figure 3. Schematic representation of the lytic infection of herpesviruses (adapted from Flint *et al*., 2000).

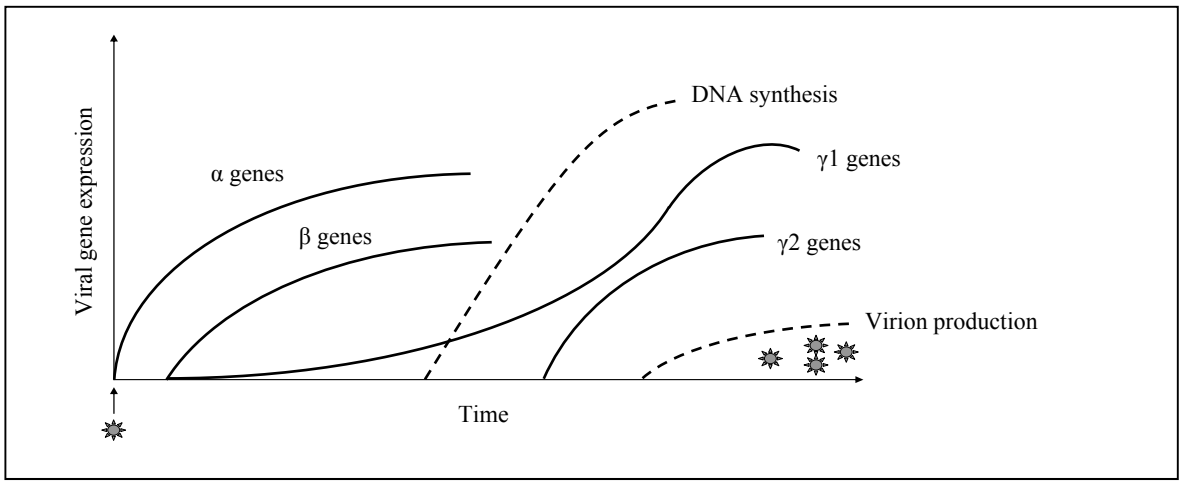


Figure 4. Kinetic of herpesvirus gene expression illustrated in a relative manner.

1997). The genome is then released and enters the nucleus through a pore of the nuclear membrane. As soon as the genome enters in the nucleus, the viral DNA circularizes prior to viral protein synthesis (Garber *et al.*, 1993). This circularization is realized by direct ligation of single unpaired 3' end nucleotides present at both ends of the genome (Davison, 1984). Tegument proteins migrate with genome into the nucleus where they regulate virus and cellular gene expression.

Herpesvirus gene expression is characterized by a transcription program where immediate-early (IE or α), early (E or β), and late (L or γ) genes are expressed successively (Figures 3 and 4) (Honess & Roizman, 1974; 1975; Jones & Roizman, 1979). IE gene expression is initiated by tegument proteins which interact with cellular transcriptional proteins, such as RNA polymerase II, to activate the transcription. IE genes encode mainly for transcription factors which inhibit IE gene expression and promote E gene expression. The maximum of E gene expression is usually observed between 4 and 8 hours post-infection (Figure 4). They are mainly coding for enzymes involved in nucleotide metabolism and viral DNA replication (Figure 3). Similarly as IE genes, E genes down regulate their own expression while stimulating the expression of L genes. Maximum L gene expression occurs after virus DNA replication (Figures 3 and 4). L genes are further divided in L1 (or γ1) and L2 (or γ2) subclasses. L1 gene expression is increased by viral DNA synthesis genes while L2 gene expression starts only after the synthesis of the viral genome (Figure 4) (Wagner *et al.*, 1998). Most of the L genes code for the proteins incorporated in mature virions; these proteins are called structural proteins. The structural proteome of a virus is defined as all the proteins which enter in the virion composition. Produced capsid proteins encoded by L genes are assembled in the nucleus to form the nucleocapsid containing newly synthesized viral DNA (Figure 3).

The replication of the viral genome is initiated from one or several origins of replication. Specific viral proteins are involved in viral DNA synthesis through a rolling-circle mechanism (Ackermann, 2004; Jacob *et al.*, 1979). This process generates concatemers consisting of complexe structure of high molecular weight made of several genomic units linked head-to-tail (Figure 3). A viral protein complex brings concatemers close to the portal complex of a capsid through which a single genomic unit is internalized and cleaved from the concatemer (Mettenleiter *et al.*, 2009).

Different models were proposed for the egress of the nucleocapsid from the nucleus to the extracellular space (Granzow *et al.*, 2001; Johnson & Spear, 1982; Wild *et al.*, 2005). In the envelopment-deenvelopment model (Figure 3), the temporary enveloped virus in the peri-nuclear space fuses with the external nuclear membrane to deliver the naked capsid in the cytoplasm. Tegument proteins are associated with the capsid before it buds into *trans*-golgi vesicles to form the envelope (Browne *et al.*, 1996; Granzow *et al.*, 2001; Masse *et al.*, 1999; Smith, 1980). The virion is finally released from the cell by exocytosis or cell lysis (Figure 3) (Flint *et al.*, 2000; Mettenleiter, 2004; Mettenleiter *et al.*, 2009). In the luminal model, the capsids bud in the internal nuclear membrane then migrate in the endoplasmic reticulum (ER). The enveloped virions are then (i) incorporated in a transport vesicle and delivered in the golgi apparatus (vesicular model) or

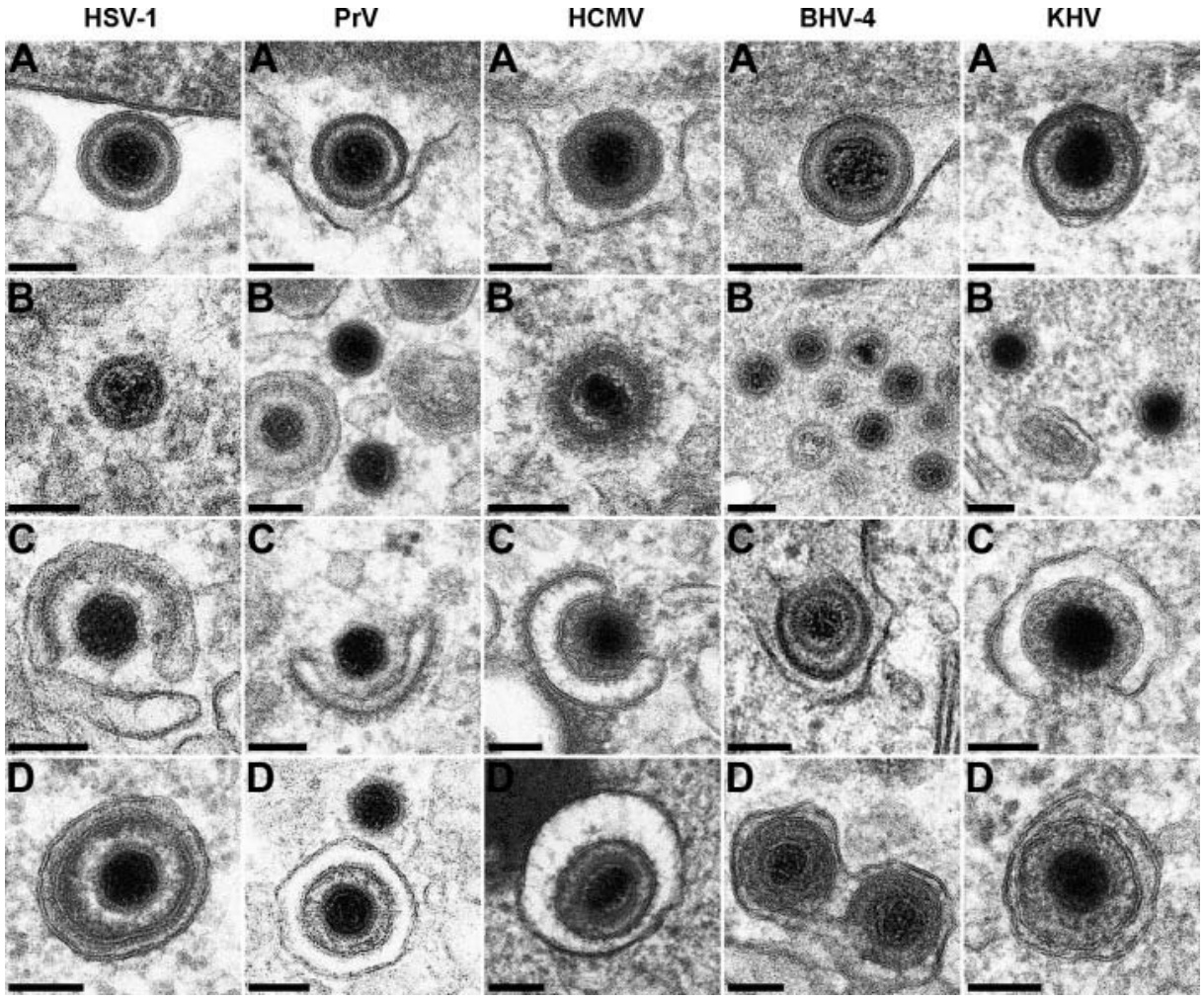


Figure 5. Acquisition process of herpesvirus envelope. (A) Primary enveloped virions in the perinuclear space. The electron-dense sharply bordered layer of tegument underlying the envelope and the absence of envelope glycoprotein spikes is noteworthy. (B) After translocation into the cytosol, capsids of HSV-1, PrV and BoHV-4 appear "naked", whereas those of HCMV and KHV are covered with a visible layer of "inner" tegument. (C) Secondary envelopment and (D) presence of enveloped virions within a cellular vesicle during transport to the plasma membrane. The same stages can be observed for members of the *Herpesviridae* family and KHV, a member of the *Alloherpesviridae* family. HSV-1: Herpes simplex type 1; PrV: Pseudorabies virus; HCMV: Human cytomegalovirus; BHV-4: Bovin herpesvirus 4; KHV: Koi herpesvirus. Bars represent 100 nm. Reproduced from Mettenleiter et al. (2009).

(ii) reach the golgi apparatus through connexions between the latter and the ER (intra-cisternal model). Independently of these models, enveloped virions are released by exocytosis (Darlington & Moss, 1968; Johnson & Spear, 1982). Recently, a new model was described for BoHV-1 where capsids present in the nucleus are able to reach the cytoplasm trough enlarged nuclear pore (Wild *et al.*, 2005). The capsids, once in the cytoplasm, bud with golgi-derived vesicles before egress from the host cell by exocytosis.

A recent study by electron microscopy on the morphogenesis of different herpesviruses belonging to the *Herpesviridae* and *Alloherpesviridae* families, concludes that the nucleocapsids follow the envelopment-deenvelopment model before being released in the extracellular space by exocytosis (Figure 5) (Mettenleiter *et al.*, 2009).

5.2 Latent infection

Latency is observed in all members of the family *Herpesviridae*. It consists in the virus maintenance in the host cell without production of viral particles. The mechanisms that induce latency are still poorly understood (Roizman & Pellet, 2007). Latency is supposed to occur when the virus infected specific cell types. The virus can then persist in the host even after the onset of an adaptive immune response able to clear cells supporting a replicative infection. Only few viral genes are expressed during latency. During latency, the genome is maintained as a non integrated episome in the nucleus. When the latent infected cells divide (if they do so), the viral episome is replicated with the cellular genomic DNA. Copies of this episome are then distributed between daughter cells. The latent infection can be interrupted by exogenous stimuli and switched to lytic infection. Latency has been studied mainly in the family *Herpesviridae*. Regulation of latency seems to be mediated mainly by transcripts (LATs for latency associated transcripts) in alphaherpesviruses (Jones, 2003) while in beta- and gammaherpesviruses latency proteins are expressed (Ballestas & Kaye, 2001; Cardin *et al.*, 2009; Lee *et al.*, 1999).

Recent studies described the presence of microRNAs (miRNA) in the genome of different herpesviruses of the *Herpesviridae* family (Pfeffer *et al.*, 2005). Ever since, several studies demonstrated miRNA productions amongst the latency transcripts (alphaherpesvirus LATs). They seem to play an important role in cooperation with the beta- and gammaherpesvirus proteins during the viral biological cycle and essentially during the latency where they can modulate cell apoptosis and immune pathways, as well as the viral lytic cycle (Burnside *et al.*, 2006; Cai *et al.*, 2005; Lu *et al.*, 2008; Umbach *et al.*, 2008; Wang *et al.*, 2008).

6. Classification of the order *Herpesvirales*

The ICTV has classified under the order *Herpesvirales* viruses encoding the putative ATPase subunit of the terminase (a complex that is responsible for packaging virus DNA into progeny capsids)

(Davison, 1992; 2002; Waltzek *et al.*, 2009). This protein is specific to herpesviruses; however, it is also conserved to a lesser degree in the T4-like bacteriophages of the family *Myoviridae* (Davison *et al.*, 2009). The *Herpesvirales* order is subdivided in three families: the *Herpesviridae*, the *Alloherpesviridae* and the *Malacoherpesviridae* (Davison *et al.*, 2009; Roizmann *et al.*, 1992).

6.1 The *Herpesviridae* family

The family *Herpesviridae* is highly studied and is divided into three sub-families: *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (Davison *et al.*, 2009; Roizman & Pellet, 2007). It regroups herpesviruses infecting reptiles, birds and mammals, including humans.

The alphaherpesviruses have a variable host range, a relatively short reproduction cycle, a rapid spread in culture, an efficient destruction of infected cells, and a capacity to establish latent infection in sensory neurons. As example, this subfamily contains the human herpesvirus 1 (HHV-1 or HSV-1) and 3 (HHV-3 or VZV), belonging to the genera *Simplexvirus* and *Varicellovirus*, respectively.

In contrast to alphaherpesviruses, betaherpesviruses have a restricted host range. The reproductive cycle is relatively long, and the infection progresses slowly in cell culture. Infected cells frequently become enlarged (cytomegalia). Their latency is established mainly in secretory glands. As example, this subfamily contains the human herpesvirus 5 (HHV-5 or HCMV) and the murid herpesvirus 1 (MuHV-1 or MCHV), belonging to the genera *Cytomegalovirus* and the *Muromegalovirus*, respectively.

Gammaherpesviruses have usually a host range restricted to the family or the order of their natural host. *In vitro*, all members replicate in lymphoblastic cells, and some also cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. As example, this subfamily contains the human herpesvirus 4 (HHV-4 or EBV) and 8 (HHV-8 or KSHV), belonging to the genera *Lymphocryptovirus* and *Rhadinovirus*, respectively.

6.2 The *Malacoherpesviridae* family

Until recently, this family consisted in a single virus (Davison *et al.*, 2005): the *Ostreid herpesvirus 1* (OsHV-1) infecting the Japanese oyster (*Crassostrea gigas*). Its genome contains 207 kb and is composed of two unique regions (U_L and U_S; 168 kb and 3 kb, respectively), each flanked by an inverted repeat (TR_L/IR_L and TR_S/IR_S of 7 kb and 10 kb, respectively). The presence of 124 ORFs are described whose 12 are duplicated in inverted repeats. Interestingly, among all these genes, 38 belong to 12 families of related genes (Davison *et al.*, 2005). Recently, a neurotropic herpesvirus infecting the gastropod abalone (*Haliotis* spp) was described (Savin *et al.*, 2010). Based on the homology existing between Abalone Herpesvirus (AbHV) and OsHV-1, it has been proposed to

Table 1. Herpesviruses of fish and amphibians (adapted from Hallon *et al.* 2011).

Virus name (abbreviation)	Clade	Common name (abbreviation)	Host(s)	Disease
Anguillid HV 1 (AngHV-1)	1	HV anguillae (HVA)	Japanese eel *Anguilla japonica* and European eel *A. Anguilla*	Haemorrhages of skin, fins, gills, liver
Cyprinid HV 1 (CyHV-1)	1	HV cyprini, carp pox HV, carp HV(CHV)	Common carp *Cyprinus carpio*	High losses in fry- exophthalmia haemorrhages, survivors have papilloma
Cyprinid HV 2 (CyHV-2)	1	Goldfish hematopoietic necrosis virus (GFHNV)	Goldfish *Carassius auratus*	High mortality at all ages. Necrosis of hematopoietic tissue, spleen, pancreas, intestine
Cyprinid HV 3 (CyHV-3)	1	Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)	Common carp	Gill inflammation, hyperplasia, and necrosis, hematopoietic tissue necrosis. High mortality at all ages
Ictalurid HV 1 (IcHV-1)	2	Channel catfish virus (CCV), Channel catfish herpesvirus	Channel catfish *Ictalurus punctatus*	Kidney, liver and intestinal necrosis, haemorrhages, high mortality in young subjects
Ictalurid HV 2 (IcHV-2)	2	Ictalurus melas HV (IcmHV)	Black bullhead *Ameiurus melas*	Kidney necrosis, haemorrhages, high mortality at all ages
Acipenserid HV 1 (AciHV-1)	2	White sturgeon HV 1	White sturgeon *Acipenser transmontanus*	diffuse dermatitis, high losses in juveniles
Acipenserid HV 2 (AciHV-2)	2	White sturgeon HV 2	White sturgeon	Epithelial hyperplasia
Salmonid HV 1 (SalHV-1)	2	HV salmonis (HPV) Steelhead herpesvirus (SHV)	Rainbow trout *Oncorhynchus mykiss*	Mild disease associated with low losses at 10 ℃. Adults: female shed virus in ovarian fluid. Asymptomatic infection
Salmonid HV 2 (SalHV-2)	2	Oncorhynchus masou virus (OMV)	Cherry salmon *O. masou*, coho salmon *O. kisutch*, sockeye salmon *O. nerka*, coho salmon *O. keta*, rainbow trout,	Viremia, external haemorrhages exophthalmia, hepatic necrosis. High mortality in young subjects. Survivors have oral papilloma. Infected female shed virus in ovarian fluid
Salmonid HV 3 (SalHV3-)	2	Epizootic epitheliotropic disease virus (EEDV)	Lake trout *Salvelinus namaycush*, lake trout × brook trout *S. fontinalis* hybrids	Epithelial hyperplasia, hypertrophy, haemorrhages on eye and jaw. High mortality in juveniles at 6–15 ℃
Gadid herpesvirus 1 (GaHV-1)	2	Atlantic cod herpesvirus (ACHV)	Atlantic cod *Gadus morhua*	Hypertrophy of cells in gills. High mortality in adults.
Ranid HV 1 (RaHV-1)	2	Luck étumor HV (LTHV)	Leopard frog *Rana pipiens*	Renal adenocarcinoma
Ranid HV 2 (RaHV-2)	2	Frog virus 4 (FV-4)	Leopard frog	No known disease
Pilchard HV	2		Australian pilchard *Sardinops sagax*	Gill inflammation associated with epithelial hyperplasia and hypertrophy. High mortality
Tilapia HV	Possible *Herpesviridae*	Tilapia larvae encephalitis virus (TLEV)	Blue tilapia *Oreochromis aureus*	Encephalitis in larvae. High mortality
Percid HV 1 (PeHV-1)		HV vitreum, walleye HV	Walleye *Stizostedion vitreum*	Diffuse epidermal hyperplasia

include the AbHV-1 in the *Malacoherpesviridae* family (Savin *et al.*, 2010). Despite the lack of similarity with the capsid proteins encoded by other herpesviruses, electron microscopy analysis demonstrates that OsHV-1 and AbHV-1 have a capsid morphology comparable to that of HHV-1 and IcHV-1 (Davison *et al.*, 2005; Savin *et al.*, 2010).

6.3 The *Alloherpesviridae* family

The *Alloherpesviridae* encompasses viruses infecting fish and amphibians. So far, this family regroups 13 viruses infecting teleost fish, 2 viruses of chondrostean fish and 2 viruses infecting amphibians (Hanson *et al.*, 2011) (Table 1). Phylogenetic studies öbased on the DNA polymerase and the terminase genes led the subdivision of the *Alloherpesviridae* family into two clades: the first clade comprises large linear dsDNA viruses (245-295 kb) as Anguillid and Cyprinid herpesviruses; the second clade comprises viruses with smaller genome (134-235 kb) as Ictalurid, Salmonid, Acipenserid and Ranid herpesviruses (Davison & Stow, 2005; Waltzek *et al.*, 2009). The genomes of several *Alloherpesviridae* have been sequenced: *Ictalurid herpesvirus 1* (IcHV-1), *Cyprinid herpesvirus 3* (CyHV-3), *Anguillid herpesvirus 1* (AngHV-1); the *Ranid herpesvirus 1* (RaHV-1) and *2* (RaHV-2). Based on these sequences, 12 conserved genes have been identified in the *Alloherpesviridae* family (Aoki *et al.*, 2007; van Beurden *et al.*, 2010).

Even though *Alloherpesviridae* are distantly related to *Herpesviridae*, there are similarities in the way they infect, replicate and persist in the host (Table 1). (i) They display a high level of host specificity, causing disease in only one species or in closely related members of the same genus. (ii) Some alloherpesviruses have been evaluated for long-term latent infections (persistence of viral DNA in survivors without production of infectious particles). Latency has been suggested in CyHV-1, CyHV-3, SalHV-2 and IcHV-1 (Hanson *et al.*, 2011). Much of our knowledge on the biology of *Alloherpesviridae* is derived from research on two models of infection: IcHV-1 for clade 2 and CyHV-3 for clade 1. CyHV-3 being the subject of this thesis, the remaining part of this introduction has been devoted to this virus.

7. References

Ackermann, M. (2004). Herpesviruses: a brief overview. *Methods Mol Biol* **256**, 199-219.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H. & Hedrick, R. P. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J Virol* **81**, 5058-5065.

Ballestas, M. E. & Kaye, K. M. (2001). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. *J Virol* **75**, 3250-3258.

Browne, H., Bell, S., Minson, T. & Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**, 4311-4316.

Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B. C., Green, P. J., Jain, N., Isaacs, G. & Morgan, R. W. (2006). Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. *J Virol* **80**, 8778-8786.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci U S A* **102**, 5570-5575.

Cardin, R. D., Schaefer, G. C., Allen, J. R., Davis-Poynter, N. J. & Farrell, H. E. (2009). The M33 chemokine receptor homolog of murine cytomegalovirus exhibits a differential tissue-specific role during in vivo replication and latency. *J Virol* **83**, 7590-7601.

Darlington, R. W. & Moss, L. H., 3rd (1968). Herpesvirus envelopment. *J Virol* **2**, 48-55.

Davison, A. J. (1984). Structure of the genome termini of varicella-zoster virus. *J Gen Virol* **65 (Pt 11)**, 1969-1977.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A. J. (2002). Evolution of the herpesviruses. *Vet Microbiol* **86**, 69-88.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J. & Thiry, E. (2009). The order Herpesvirales. *Arch Virol* **154**, 171-177.

Davison, A. J. & Stow, N. D. (2005). New genes from old: redeployment of dUTPase by herpesviruses. *J Virol* **79**, 12880-12892.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., Le Deuff, R. M. & Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86**, 41-53.

Dimmock, N. J., Easton, A. J. & Leppard, K. N. (2007). Towards a definition of a virus. In *Introduction to modern virology*, 6 edn, pp. 3 - 17. Edited by N. J. Dimmock, A. J. Easton & K. N. Leppard. Oxford: Wiley-Blackwel.

Flint, S., Racaniello, V. R., Enquist, L. W., Skalka, A. M. & Krug, R. M. (2000). *Virology: molecular biology, pathogenesis, and control*. Washington, D. C: ASM press.

Garber, D. A., Beverley, S. M. & Coen, D. M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* **197**, 459-462.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N. & Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**, 3675-3684.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**, 8-19.

Honess, R. W. & Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc Natl Acad Sci U S A* **72**, 1276-1280.

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-457.

Johnson, D. C. & Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* **43**, 1102-1112.

Jones, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16**, 79-95.

Jones, P. C. & Roizman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. *J Virol* **31**, 299-314.

King, A., Lefkowitz, E., Adams, M. & Carstens, E. (2012). Order of presentation of virus taxonomic descriptions. In *Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 23-36. Edited by A. King, E. Lefkowitz, M. Adams & E. Carstens. London: Elsevier.

Lee, M. A., Diamond, M. E. & Yates, J. L. (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**, 2974-2982.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M. & Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* **82**, 10436-10443.

Masse, M. J., Jons, A., Dijkstra, J. M., Mettenleiter, T. C. & Flamand, A. (1999). Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. *J Virol* **73**, 10503-10507.

McGeoch, D. J., Davison, A. J., Dolan, A., Gatherer, D. & Sevilla-Reyes, E. E. (2008). Molecular evolution of the Herpesvirales. In *Origin and evolution of viruses*, 2 edn, pp. 447-476. Edited by E. Domingo, C. R. Parrish & J. J. Holland. Amsterdam: Elsevier.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-180.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M. & Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods* **2**, 269-276.

Roizman, B. & Pellet, P. E. (2007). The Family: Herpesviridae a brief introduction. In *Fields virology*, 5 edn, pp. 2479-2499. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams and Wilkins.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. & Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**, 425-449.

Savin, K. W., Cocks, B. G., Wong, F., Sawbridge, T., Cogan, N., Savage, D. & Warner, S. (2010). A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J* **7**, 308.

Smith, J. D. (1980). An additional role for the outer nuclear membrane in the morphogenesis of herpes simplex virus. *Intervirology* **13**, 312-316.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007-1021.

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-410.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780-783.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

Wagner, E. K., Petroski, M. D., Pande, N. T., Lieu, P. T. & Rice, M. (1998). Analysis of factors influencing kinetics of herpes simplex virus transcription utilizing recombinant virus. *Methods* **16**, 105-116.

Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J. & Hedrick, R. P. (2009). Phylogenetic relationships in the family Alloherpesviridae. *Dis Aquat Organ* **84**, 179-194.

Wang, F. Z., Weber, F., Croce, C., Liu, C. G., Liao, X. & Pellett, P. E. (2008). Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *J Virol* **82**, 9065-9074.

Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepfe, E., Ackermann, M., Mueller, M. & Walther, P. (2005). Impairment of nuclear pores in bovine herpesvirus 1 infected MDBK cells. *J Virol* **79**, 1071-1083.

Introduction

2nd chapter:

Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research

Veterinary Research (2013), 44:85

Krzysztof Rakus[1], Ping Ouyang[1], Maxime Boutier[1], Maygane Ronsmans[1], Anca Reschner[1], Catherine Vanczok[1], Joanna Jazowiecka-Rakus[1]and Alain Vanderplasschen[1]*

[1]Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium

* Corresponding author. E-mail: a.vdplasschen@ulg.ac.be. Phone: +32 4 366 42 64

Abstract

Cyprinid herpesvirus 3 (CyHV-3), a member of the family *Alloherpesviridae* is the causative agent of a lethal, highly contagious and notifiable disease in common and koi carp. The economic importance of common and koi carp industries together with the rapid spread of CyHV-3 worldwide, explain why this virus became soon after its isolation in the 1990s a subject of applied research. In addition to its economic importance, an increasing number of fundamental studies demonstrated that CyHV-3 is an original and interesting subject for fundamental research. In this review, we summarized recent advances in CyHV-3 research with a special interest for studies related to host-virus interactions.

Key words: Cyprinid herpesvirus 3, CyHV-3, koi herpesvirus, KHV, *Alloherpesviridae*, Common carp, host-virus interactions

1. Introduction

The common carp (*Cyprinus carpio*) is one of the oldest cultivated fish species. In China, culture of carp dates back to at least the 5th century BC, whereas in Europe, carp farming began during the Roman Empire [1]. Nowadays, common carp is one of the most economically valuable species in aquaculture: (*i*) it is one of the main cultivated fish for human consumption with a world production of 3.4 million tons per year [2]; (*ii*) it is produced and stocked into fishing areas for angling purpose; and (*iii*) its colorful, ornamental varieties (koi carp) grown for personal pleasure and competitive exhibitions represent probably the most expensive market of individual freshwater fish with some prize-winners sold for 10^4-10^6 US dollars [3].

Herpesviruses infect a wide range of vertebrates and invertebrates [4]. However, the host-range of individual herpesvirus species is generally restricted revealing host-virus co-evolution. In aquaculture, herpesvirus infections have been associated with mass mortality of different fish species causing severe economic losses [5-7]. In the late 1990s, a new highly contagious and virulent disease began to cause severe economic losses in both koi and common carp industries. Soon after its first known occurrences reported in Israel, USA, and Germany [8, 9], the disease was described in various countries worldwide. The rapid spread of the disease was attributed to international fish trade and to koi shows around the world [10]. The causative agent of the disease was initially called koi herpesvirus (KHV) because of its morphological resemblance to viruses of the order *Herpesvirales* [9]. The virus was subsequently called carp interstitial nephritis and gill necrosis virus (CNGV) because of the associated lesions [11]. Finally, on the basis of genome homology with previously described cyprinid herpesviruses the virus was renamed cyprinid herpesvirus 3 (CyHV-3) [12].

Because of its worldwide spread and the economic losses it caused, CyHV-3 became rapidly a notifiable disease and a subject of application oriented research. However, an increasing number of recent studies have demonstrated that it is also an interesting subject for fundamental research. In this review, we summarized recent advances in CyHV-3 research with a special interest for those related to host-virus interactions.

2. Characterization of CyHV-3

2.1 General description

2.1.1 Classification

CyHV-3 is a member of genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales* (Figure 1a) [13]. The *Alloherpesviridae* is a newly designated family which regroups herpesviruses infecting fish and amphibians [14]. It is divided into four genera: *Cyprinivirus*, *Ictalurivirus*, *Salmonivirus*, and *Batrachovirus* [13]. The genus *Cyprinivirus* contains viruses that infect common carp (Cyprinid herpesvirus 1 and 3; CyHV-1 and CyHV-3), goldfish (Cyprinid herpesvirus 2; CyHV-2)

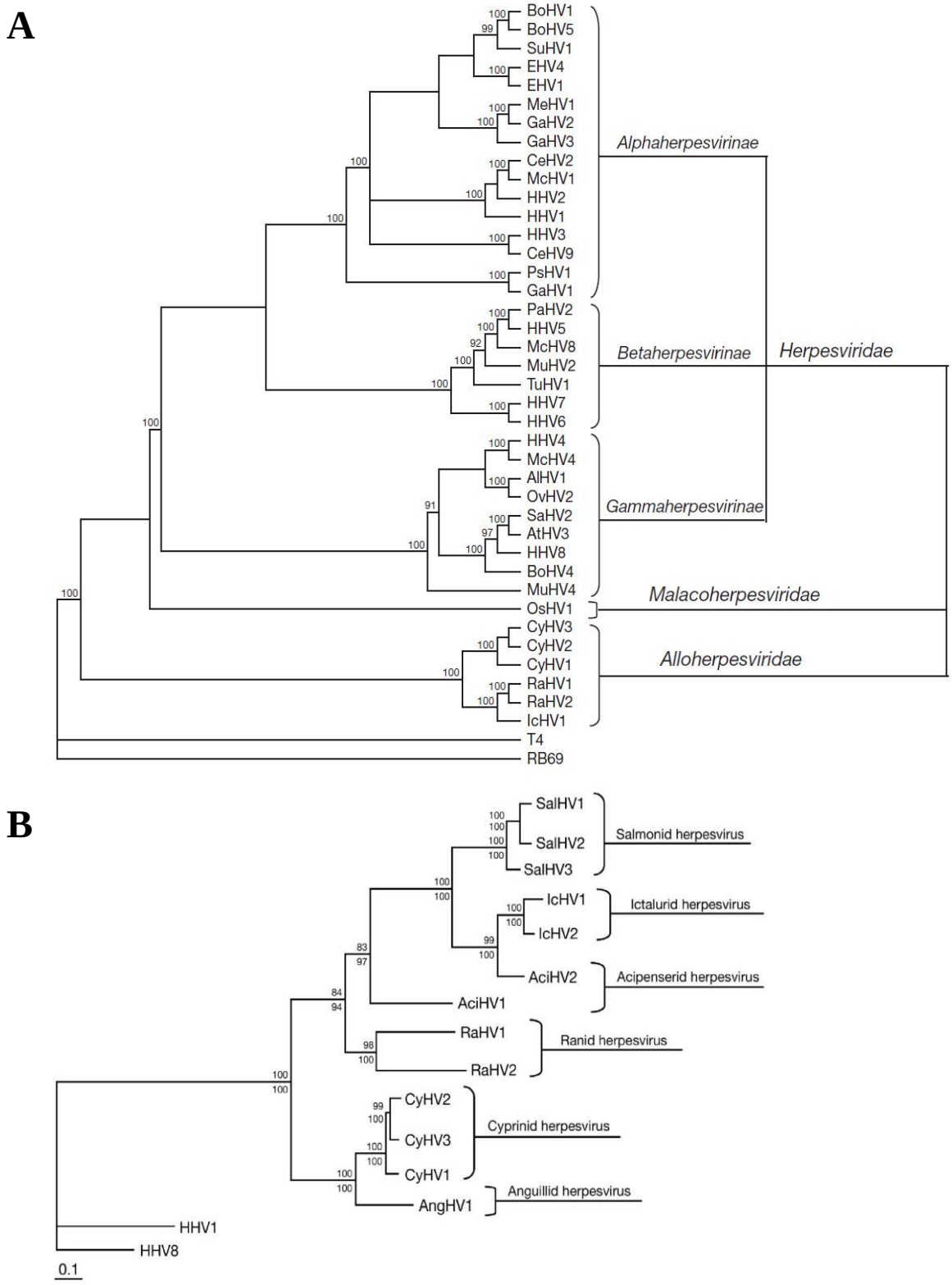


Figure 1. Phylogeny of the order *Herpesvirales* and the *Alloherpesviridae* family. (A) Cladogram depicting relationships among viruses in the order *Herpesvirales*, based on the conserved regions of the terminase gene. The Bayesian maximum likelihood tree was rooted using bacteriophages T4 and RB69. Numbers at each node represent the posterior probabilities (values > 90 are shown) of the Bayesian analysis. **(B)** Phylogenetic tree depicting the evolution of fish and amphibian herpesviruses, based on sequences of the DNA polymerase and terminase genes. The maximum likelihood tree was rooted with two mammalian herpesviruses (HHV-1 and HHV-8). Maximum likelihood values (> 80 are shown) and Bayesian values (> 90 are shown) are indicated above and below each node, respectively. Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. AlHV-1: alcelaphine herpesvirus 1; AtHV-3: ateline herpesvirus 3; BoHV-1, -4, -5: bovine herpesvirus 1, 4, 5; CeHV-2, -9: cercopithecine herpesvirus 2, 9; CyHV-1, -2: cyprinid herpesvirus 1, 2; EHV-1, -4: equid herpesvirus 1, 4; GaHV-1, -2, -3: gallid herpesvirus 1, 2, 3; HHV-1, -2, -3, -4, -5, -6, -7, -8: human herpesvirus 1, 2, 3, 4, 5, 6, 7, 8; IcHV-1: ictalurid herpesvirus 1; McHV-1, -4, -8: macacine herpesvirus 1, 4, 8; MeHV-1: meleagrid herpesvirus 1; MuHV - 2, - 4: murid herpesvirus 2, 4; OsHV-1: ostreid herpesvirus 1; OvHV-2: ovine herpesvirus 2; PaHV-1: panine herpesvirus 1; PsHV-1: psittacid herpesvirus 1; RaHV-1, -2: ranid herpesvirus 1, 2; SaHV - 2: saimiriine herpesvirus 2; SuHV-1: suid herpesvirus 1; TuHV-1: tupaiid herpesvirus 1. Reproduced with permission from Waltzek et al. [14].

and freshwater eel (Anguillid herpesvirus 1; AngHV-1). Phylogenetic analyses revealed that the genus *Cyprinivirus* forms a clade distinct from the three other genera listed above (Figure 1b). Viruses of the *Cyprinivirus* genus possess the largest genomes (248–295 kb) in the order *Herpesvirales*.

2.1.2 Morphology

Like all members of the order *Herpesvirales*, CyHV-3 virions are composed of an icosahedral capsid containing the genome, a lipid envelope bearing viral glycoproteins and an amorphous layer of proteins termed tegument, which resides between the capsid and the envelope [15]. The diameter of CyHV-3 virions is 167–200 nm according to the infected cell type (Figure 2) [15]. Morphogenesis of CyHV-3 is also characteristic of the order *Herpesvirales*, with assembly of the nucleocapsid and acquisition of the lipid envelope (derived from host cell trans-golgi membrane) that take place in the nucleus and the cytosol of the host cell, respectively [9,15,16].

2.1.3 Genome

The genome of CyHV-3 is a 295 kb, linear, double stranded DNA molecule consisting of a large central portion flanked by two 22 kb repeat regions, called the left and right repeats [18]. To date, this is the largest genome among all sequenced herpesviruses. The CyHV-3 genome has been cloned as a stable and infectious bacterial artificial chromosome (BAC), which can be used to produce CyHV-3 recombinants [19].

The CyHV-3 genome is predicted to contain 155 potential protein-coding open reading frames (ORFs), among which eight (ORF1-ORF8) are duplicated in terminal repeats [13]. Nine ORFs are characterized by the presence of introns [13]. CyHV-3 genome encodes five gene families: ORF2, tumor necrosis factor receptor (TNFR), ORF22, ORF25, and RING gene families [18]. The ORF25 family consists of 6 paralogous sequences (ORF25, ORF26, ORF27, ORF65, ORF148 and ORF149) encoding potential type 1 membrane glycoproteins. Independently of the viral strain sequences, ORF26 is described as a pseudogene; while ORF27 has been characterized as pseudogene in 2 out of 3 sequenced laboratory strains [18]. All non-fragmented members of this family (ORF25, ORF65, ORF148 and ORF149) are incorporated in mature virions, presumably in the envelope [20].

Interestingly, CyHV-3 genome encodes proteins potentially involved in immune evasion mechanisms such as, for example, G-protein coupled receptor (encoded by ORF16), TNFR homologues (encoded by ORF4 and ORF12) and an interleukine-10 (IL-10) homologue (encoded by ORF134) [18].

Among the family *Alloherpesviridae*, twelve ORFs (named core ORFs) are conserved in all sequenced viruses and were presumably inherited from a common ancestor [13]. The Cyprinivirus (CyHV-1, CyHV-2, and CyHV-3) possess 120 orthologous ORFs. Twenty one ORFs are unique to CyHV-3, including ORF134 encoding an IL-10 homolog [13]. The recently described second IL-10

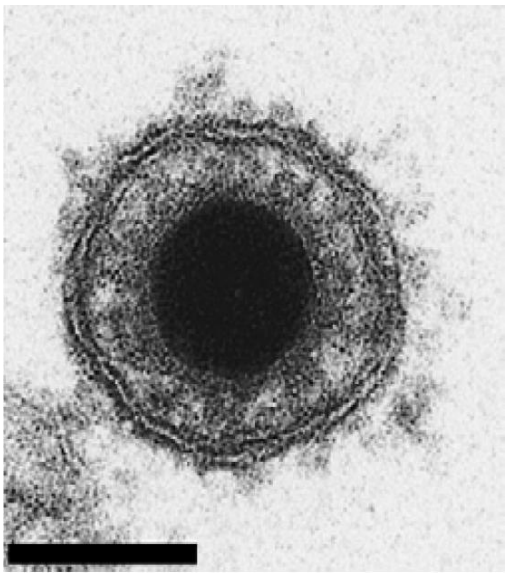


Figure 2. Electron microscopy examination of CyHV-3 virion. Bar represents 100 nm. Adapted with permission from Mettenleiter et al. [17].

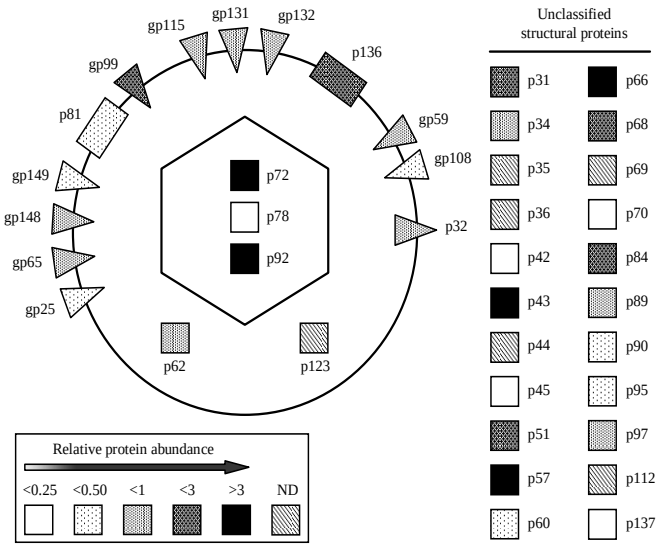


Figure 3. Schematic representation of CyHV-3 virion proteome. The viral composition of the envelope (circle), capsid (hexagon) and tegument is indicated. Membrane proteins of type 1, 2 and 3 are represented by triangles pointed inside, triangles pointed outside and rectangles, respectively. Other proteins are shown as squares. The different fillings indicate the relative abundance of proteins based on their emPAI (< 0.25, < 0.50, < 1, < 3 and > 3). p: protein, gp: glycoprotein, ND: no data. Reproduced with permission from Michel et al. [20].

homolog in the family *Alloherpesviridae* encoded by AngHV-1 does not seem to be an orthologue of the CyHV-3 ORF134 [21]. CyHV-3 shares 40 orthologous ORFs with AngHV-1 although the total number of ORFs shared by all CyHVs with AngHV-1 is estimated to be 55 [13]. This supports the phylogenetic conclusion that among the genus *Cyprinivirus*, CyHVs are more closely related to each other than to other members of the family *Alloherpesviridae* [14]. Interestingly, CyHV-3 also encodes genes with closest relatives in viral families such as *Poxviridae* and *Iridoviridae* [18, 22].

2.1.4 Genotypes

Whole genome analysis of three CyHV-3 strains isolated in Israel (CyHV-3 I), Japan (CyHV-3 J) and United States (CyHV-3 U) revealed high sequence identity between the strains [18]. The relationships between these strains revealed that U and I strains are more closely related to each other and form one lineage (U/I), whereas J strain is more distinct and forms a second lineage (J) [18]. The existence of genetic differences between European lineage (including U and I genotypes) and Asian lineage (including J genotype) were later confirmed and suggests independent CyHV-3 introductions in various geographical locations [23, 24]. Furthermore, Kurita *et al.* demonstrated that the Asian lineage contains only two variants (A1 and A2) while the European lineage has seven variants (E1–E7) [24]. Recently, a new intermediate genetic lineage of CyHV-3 including isolates from Indonesia has been suggested [25]. This hypothesis was later supported by analyses of multi-locus variable number of tandem repeats (VNTR). These analyses also suggested that genetically distinct viral strains can coexist in a same location following various introduction events [26]. Although previous study described presence of both CyHV-3 lineages in Europe [23], an European genotype of CyHV-3 has only been revealed recently in East and Southeast Asia [27]. Recently, Han *et al.* described polymorphism in DNA sequences encoding three envelope glycoprotein genes (ORF25, ORF65, and ORF116) among CyHV-3 strains from different geographical origins [28].

2.1.5 Proteome

Different groups used mass spectrometry to identify CyHV-3 proteins and to study their interactions with cellular and viral proteins. The structural proteome of CyHV-3 was recently characterized by using liquid chromatography tandem mass spectrometry [20]. A total of 40 structural proteins, comprising 3 capsid, 13 envelope, 2 tegument, and 22 unclassified proteins, were described (Figure 3). The genome of CyHV-3 possesses 30 potential transmembrane-coding ORFs [18]. With the exception of ORF81, which encodes a type 3 membrane protein expressed on the CyHV-3 envelope, no CyHV-3 structural proteins have been studied [20, 29]. ORF81 is thought to be one of the most immunogenic (major) membrane proteins of CyHV-3 [29]. Recently, Gotesman *et al.* using anti-CyHV-3 antibody-based purification coupled with mass spectrometry, identified 78 host proteins and five potential immunogenic viral proteins [30]. In another study, concentrated supernatant was

produced from CyHV-3 infected CCB cultures and analyzed by 2D-LC MS/MS in order to identify CyHV-3 secretome. Five viral and 46 cellular proteins were detected [31]. CyHV-3 ORF12 and ORF134 encoding respectively a soluble TNFR homologue and an IL-10 homologue, were among the most abundant secreted viral proteins [31].

2.2 *In vitro* replication

CyHV-3 is widely cultivated in cell lines derived from common carp brain (CCB), gills (CCG) and fin (CaF-2) [32, 33]. Permissive cell lines have also been derived from koi fin: KF-1 [9], KFC [11], KCF-1 [34], NGF-2 and NGF-3 [16]. Non-carp cell lines, such as silver carp fin (Tol/FL) and goldfish fin (Au) were also described as permissive to CyHV-3 [35]. Oh *et al.* reported the expression of cytopathic effect (CPE) in cell line from fathead minnow (FHM) after inoculation with CyHV-3 [36], but this observation was not confirmed by other studies [9, 35].

In vitro study showed that all annotated CyHV-3 ORFs are transcribed during CyHV-3 replication [37]. Transcription of CyHV-3 genes starts as early as 1 h post-infection and viral DNA synthesis initiates as early as 4–8 h post-infection [37]. Similar to all other herpesviruses, most of CyHV-3 ORF transcripts can be classified into three temporal kinetic classes: immediate early (IE; n = 15 ORFs), early (E; n = 111 ORFs) and late (L; n = 22 ORFs). Seven ORFs are unclassified [37]. Fuchs *et al.* demonstrated that CyHV-3 ORFs that encode for three enzymes implicated in nucleotide metabolisms: thymidine kinase (ORF55), dUTPase (ORF123) and ribonucleotide reductase (ORF141) are nonessential for virus replication *in vitro* [38].

2.3 Temperature restriction

Water temperature is one of the major environmental factors that influences the onset and severity of viral infection in fish [39]. This statement certainly applies to CyHV-3 as temperature was shown to affect drastically both viral replication *in vitro* and CyHV-3 disease *in vivo*.

2.3.1 *In vitro*

CyHV-3 replication in cell culture is restricted by temperature. Optimal viral growth in KF-1 cell line was observed at temperatures between 15°C and 25°C. Virus propagation and virus gene transcription are gradually turned off when cells are moved from permissive temperature to the non-permissive temperature of 30°C [40, 41]. However, infected cells maintained for 30 days at 30°C preserve infectious virus, as demonstrated by viral replication when the cells are returned to permissive temperatures [40].

2.3.2 *In vivo*

CyHV-3 disease occurs naturally when water temperature is between 18°C and 28°C. Several studies demonstrated that transfer of recently infected fish (between 1 and 5 days post-infection (dpi)) to non-permissive low (≤ 13°C) or high temperatures (> 30°C) significantly reduces the mortality [11, 42-44]. Water temperature was also shown to affect the onset of mortality: the first mortalities occurred between 5–8 and 14–21 dpi when the fish were kept between 23-28°C and 16-18°C, respectively [42,45].

2.4 Geographical distribution

CyHV-3 was first isolated from infected koi originating from Israel and USA in 2000 [9]. Soon after, outbreaks of CyHV-3 occurred in many countries in Europe, Asia and Africa [10, 22]. Currently, only South America, Australia and northern Africa seem to be free of CyHV-3. The global and rapid spread of the virus is thought to be mainly due to the international trading of common and koi carp, but also to koi shows.

2.5 Presence of CyHV-3 in natural environment

In addition to its economic impact on common and koi carp industries, CyHV-3 has also a negative environmental impact by affecting wild populations of carp. In 2003, the first outbreak of CyHV-3 disease among wild carp occurred in the Yoshi river in Japan [46]. The virus then spread among several freshwater systems and caused mass mortalities in wild carp populations. In Lake Biwa, about 70% of carp population (more than 100 000 fish) died due to CyHV-3 infection in 2004 [46]. Mass mortalities of wild carp have been also described in angling waters in UK in 2003 [47], in New York and South Carolina, USA in 2004 [48, 49] and in Kawartha Lakes region, Ontario, Canada in 2007 [50]. The monitoring of the distribution of CyHV-3 in rivers and lakes in Japan demonstrated that it can persist in the wild carp populations and can be subsequently transmitted to naïve fish [46, 51, 52]. Studies performed in habitats with CyHV-3 history suggested that sediments [53] and aquatic invertebrates feeding by water filtration could represent potential reservoirs of CyHV-3 [54]. Moreover the viral DNA could be detected in water not only during the outbreak of the disease but also for at least 3 months after the end of mass mortality [51]. However, it has to be noted that these studies relied on the detection of viral genome and not CyHV-3 infectivity. Consequently further studies are required to determine whether these potential reservoirs of infectious virus could play a role in CyHV-3 epidemiology.

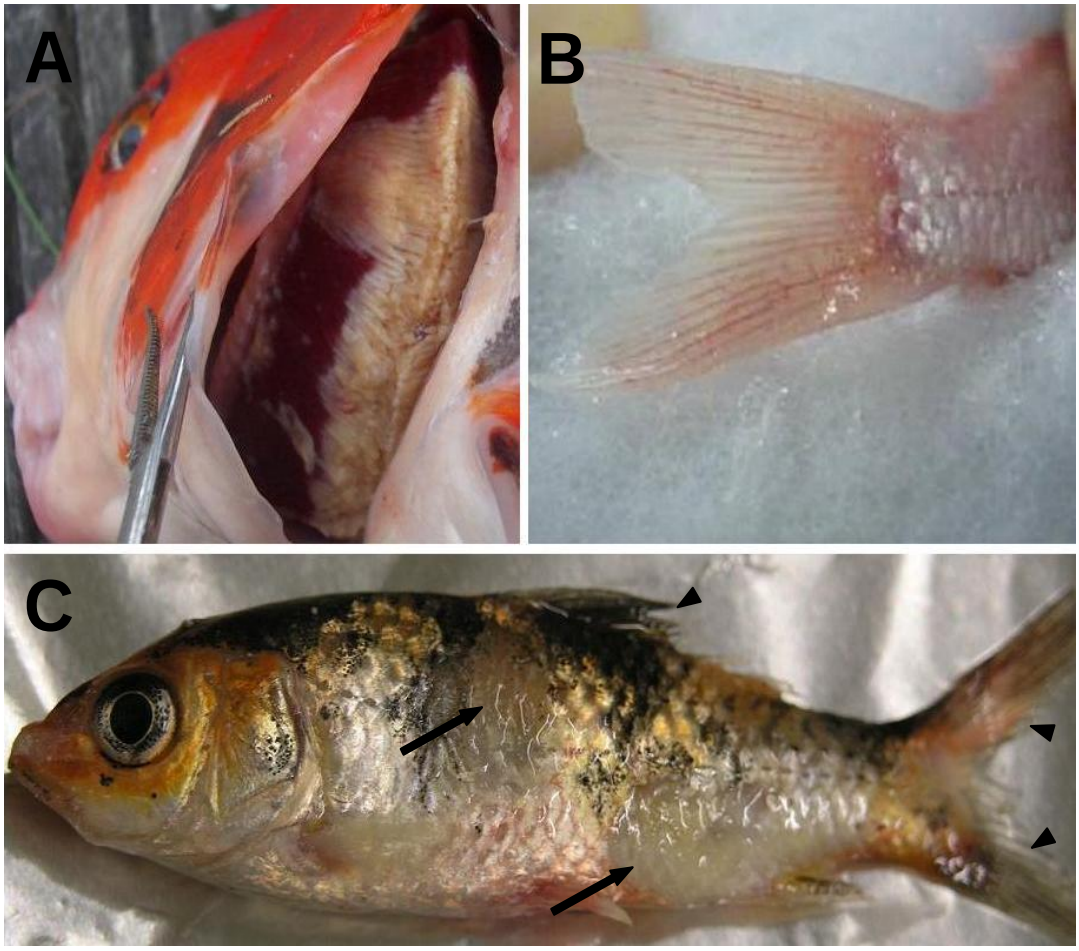


Figure 4. Some of the clinical signs observed during CyHV-3 infection. (A) Severe gill necrosis. **(B)** Hyperemia at the base of the caudal fin. **(C)** Herpetic skin lesions on the body (arrows) and fin erosion (arrowheads). Reproduced with permission from Michel et al. [22].

3. Disease

3.1 Disease characteristics

CyHV-3 disease is seasonal, occurring when water temperature is between 18°C and 28°C. It is restricted to common and koi carp and their hybrids with other species [55]. It is highly contagious and extremely virulent with mortality rate that can reach 80 to 100%. Fish infected with CyHV-3 by immersion, injection or oral route and kept at 23-28°C die between 5 and 22 dpi with a peak of mortality between 8 and 12 dpi [9, 56, 57]. Gilad *et al.* suggested that loss of osmoregulation of the gills, gut and kidney contributes to mortality during acute infection with CyHV-3 [42]. Furthermore, CyHV-3 infected fish are more susceptible to secondary infections by bacterial, parasitic or fungal pathogens which may cause further mortality within the population.

3.1.1 Clinical signs

The first clinical signs appear at 2–3 dpi. Fish become lethargic, lie at the bottom of the tank with the dorsal fin folded and exhibit loss of appetite. In ponds, infected fish are usually gathering close to the water inlet or sides of the pond and gasp at the surface of water. Gill necrosis coupled with extensive discoloration and increased mucus secretion appear as early as 3 dpi. Depending on the stage of the infection, the skin exhibits different clinical signs, such as hyperemia, particularly at the base of the fins and on the abdomen; pale, irregular patches on the skin associated with mucus hypersecretion at the beginning of infection; peeling away of dead epithelium and lack of mucus cover in the later stage of infection; appearance of epidermis surface with a sandpaper-like texture; and herpetic lesions (Figure 4). In addition, fin erosion and bilateral enophthalmia (sunken eyes) are observed in the later stages of infection. Some fish show neurologic signs in the final stage of disease, when they become disoriented and lose equilibrium [9, 10, 58].

3.1.2 Histopathology

The most important histopathological changes are observed in the gills. They involve erosion of primary lamellae, fusion of secondary lamellae and adhesion of gill filaments [58, 59]. Gills also exhibit hyperplasia, hypertrophy and/or nuclear degeneration of branchial epithelium and congestion of the blood vessels in the gill arch [15, 59]. Severe inflammation and gill necrosis resulting in the complete loss of lamellae can also be observed [31, 59]. In the kidney, the hematopoietic cells are the most affected ones [15]. However, a weak peritubular inflammatory infiltrate is evident in kidney as early as 2 dpi and increases with time. It is accompanied by blood vessel congestion and degeneration of the tubular epithelium in many nephrons [59]. In the spleen and liver, the most obviously infected cells are splenocytes and hepatocytes, respectively [15]. In the liver, mild inflammatory infiltrates are observed mainly in the parenchyma [59]. In the brain, focal meningeal and parameningeal

Table 1. Organisms tested for CyHV-3 infection

Common name (species)	Detection of CyHV-3			Detection of CyHV-3 genome in naïve carp after cohabitation
	DNA	Transcript	Antigen	
Vertebrates				
Cyprinidae				
Goldfish (*Carassius auratus*)	Yes [62,68-70]	Yes [68]	Yes [69]	Yes [68-70]
Ide (*Leuciscus idus*)	Yes [62,63]	NT	NT	NT
Grass carp (*Ctenopharyngodon idella*)	Yes [62,64,70]	NT	NT	Yes [64,70]
Silver carp (*Hypophthalmichthys molitrix*)	Yes [64,70]	NT	NT	Yes [64,70]
Prussian carp (*Carassius gibelio*)	Yes [64,70]/ No [65]	NT	NT	Yes [70]/No [65]
Crucian carp (*Carassius carassius*)	Yes [64]	NT	NT	NT
Tench (*Tinca tinca*)	Yes [64,65,70]	NT	NT	Yes [64,65,70]
Vimba (*Vimba vimba*)	Yes [63,64]	NT	NT	Yes [64]
Common bream (*Abramis brama*)	Yes [64,65]	NT	NT	Yes [64]
Common roach (*Rutilus rutilus*)	Yes [64,65]	NT	NT	Yes [64]/No [65]
Common dace (*Leuciscus leuciscus*)	Yes [64,65]	NT	NT	No [65]
Gudgeon (*Gobio gobio*)	Yes [64,65]	NT	NT	Yes [65]
Rudd (*Scardinius erythrophthalmus*)	Yes [65]	NT	NT	Yes [65]
European chub (*Squalius cephalus*)	Yes[64]/No[65]	NT	NT	NT
Common barbel (*Barbus barbus*)	Yes [64]	NT	NT	NT
Belica (*Leucaspius delineatus*)	Yes [64]	NT	NT	NT
Common nase (*Chondrostoma nasus*)	Yes [64]	NT	NT	NT
Acipenseridae				
Russian sturgeon (*Acipenser gueldenstaedtii*)	Yes [66]	NT	NT	NT
Atlantic sturgeon (*Acipenser oxyrhynchus*)	Yes [66]	NT	NT	NT
Cobitidae				
Spined loach (*Cobitis taenia*)	Yes [64]	NT	NT	NT
Cottidae				
European bullhead (*Cottus gobio*)	Yes [64]	NT	NT	NT
Esocidae				
Northern pike (*Esox lucius*)	Yes [64,65]	NT	NT	Yes [65]
Gasterosteidae				
Three-spined stickleback (*Gasterosteus aculeatus*)	Yes [65]	NT	NT	No [65]
Ictaluridae				
Brown bullhead (*Ameiurus nebulosus*)	Yes [65]	NT	NT	No [65]
Loricariidae				
Ornamental catfish (*Ancistrus sp.*)	Yes [62]	NT	NT	NT
Percidae				
European perch (*Perca fluviatilis*)	Yes [64,65]	NT	NT	Yes [64]/No [65]
Ruffe (*Gymnocephalus cernua*)	Yes[64]/No [65]	NT	NT	Yes [64,65]
Invertebrates				
Swan mussels (*Anodonta cygnea*)	Yes [54]	NT	NT	NT
Scud (*Gammarus pulex*)	Yes [54]	NT	NT	NT

NT- not tested.

inflammation is observed [59]. Analysis of brain from fish that showed clear neurologic signs revealed congestion of capillaries and small veins associated with edematous dissociation of nerve fibers in the valvula cerebelli and medulla oblongata [15]. In the skin, the number of goblet cells is reduced by 50% in infected fish. Furthermore, the goblet cells appeared mostly slim and slender which suggests that mucus was released and had not been replenished. In addition, erosion of skin epidermis is frequently observed [60].

3.2 Host range and susceptibility

CyHV-3 causes a symptomatic disease only in common and koi carp. Hybrids of koi × goldfish and koi × crucian carp are also affected by CyHV-3 disease, with mortality rate of 35% and 91%, respectively [55]. Common carp × goldfish hybrids have also been reported to show some susceptibility to CyHV-3 infection; however, the mortality rate observed was rather limited (5%) [61]. PCR detection of CyHV-3 performed on cyprinid and non-cyprinid fish species, but also on freshwater mussels and crustaceans, suggested that these species could act as reservoirs of the virus (Table 1) [54, 62-67]. Cohabitation experiments suggest that some of these fish species (goldfish, tench, vimba, common bream, common roach, European perch, ruffe, gudgeon, rudd, northern pike, silver carp and grass carp) can carry CyHV-3 asymptomatically and transmit it to naïve carp [64, 65, 68-70]. Consistent with this observation, *in vitro* studies showed that CyHV-3 can replicate and cause CPE in cell cultures derived not only from common and koi carp but also from silver carp and goldfish [35]. Recent studies provided increasing evidence that CyHV-3 can infect goldfish asymptomatically [68, 69]. Finally, the World Organisation for Animal Health (OIE) listed four CyHV-3 susceptible species (*Cyprinus carpio* and its hybrids, goldfish, Russian sturgeon and Atlantic sturgeon) and two potential susceptible species (grass carp and ide) [71].

Carp of all ages, from juveniles upwards, are affected by CyHV-3, but younger fish (1-3 months, 2.5-6 g) seem to be more susceptible to infection than mature fish (1 year, ≈230 g) [58]. Ito *et al.* suggested that carp larvae are not susceptible to CyHV-3 since larvae (3 days post-hatching) infected with virus showed no mortality whereas most of the carp juveniles (>13 days post-hatching) died after infection [72]. However, recent study using CyHV-3 recombinant strain expressing luciferase (LUC) as a reporter gene, demonstrates that carp larvae are sensitive and permissive to CyHV-3 infection immediately after hatching and that their sensitivity increases with the developmental stages [73].

3.3 Pathogenesis

In early reports, it has been suggested that CyHV-3 may enter the host through infection of the gills based on detection of viral particles and viral genome in this organ as early as 1–2 dpi [42, 59]. However, more recent studies using *in vivo* bioluminescent imaging system demonstrated that

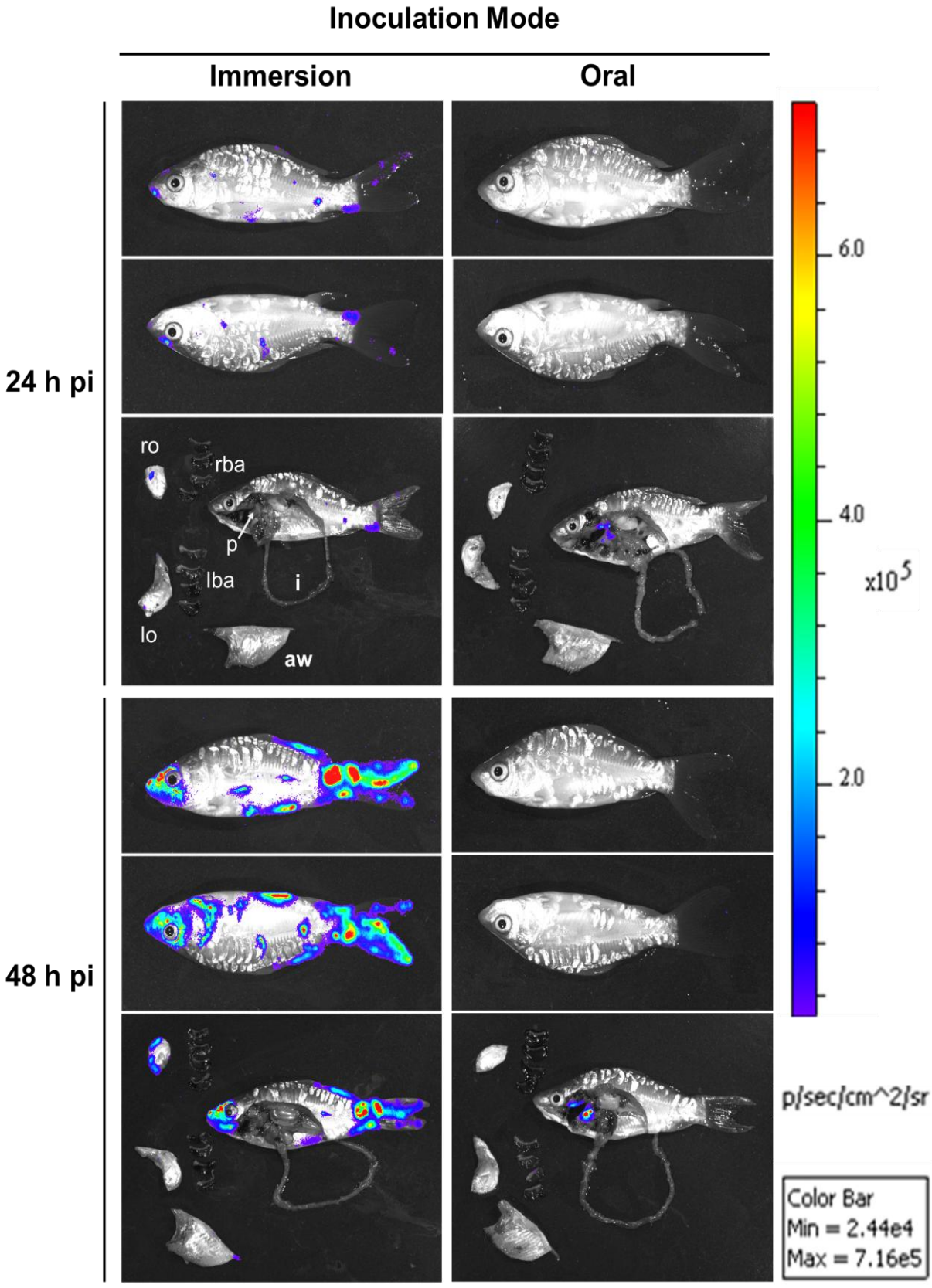


Figure 5. The portal of entry of CyHV-3 in carp analysed by bioluminescence imaging. Two groups of fish (mean weight 10 g) were infected with a recombinant CyHV-3 strain expressing luciferase as a reporter gene either by bathing them in water containing the virus (Immersion, left column) or by feeding them with food pellets contaminated with the virus (Oral, right column). At the indicated time post-infection, six fish per group were analysed by bioluminescence IVIS. Each fish was analysed lying on its right and its left side. To analyze internal signals, fish were euthanized and dissected immediately after in vivo bioluminescence imaging. Dissected fish and isolated organs were analysed for ex vivo bioluminescence. The analysis of one fish is presented for each time point and inoculation mode. Pictures collected over the course of this experiment are presented with a standardized minimum and maximum threshold value for photon flux. rba, right branchial arches; lba, left branchial arches; ro, right operculum; lo, left operculum; p, pharynx; aw, abdominal wall; i, intestine. Reproduced with permission from Fournier et al. [57].

according to epidemiological conditions CyHV-3 can enter carp either by skin (immersion in infectious water) or pharyngeal periodontal mucosa infection (ingestion of infectious materials) (Figure 5) [57, 74]. The epidermis of teleost fish is a living stratified squamous epithelium that is capable of mitotic division at all levels (even the outermost squamous layer). The scales are dermal structures and consequently are covered by the epidermis [74]. Removal of skin mucus and epidermal lesions facilitates the entry of virus into the host (Figure 6) [75]. After initial replication in the epidermis [74] the virus is spreading rapidly in infected fish as indicated by detection of CyHV-3 DNA in almost all internal tissues as early as 24 h post-infection [42]. The tropism of CyHV-3 for white blood cells most probably explains such a rapid spread of the virus within the body [76]. Virus replication in organs such as the gills, skin and gut represents source of viral excretion into the water. Recently, pharyngeal periodontal mucosa has been shown to be the portal of entry of CyHV-3 after infection by the oral route using food pellets contaminated with the virus [57]. This model of inoculation led to the spreading of the infection to the various organs tested as well as resulted in clinical signs and mortality rate comparable to the infection by immersion [57].

All members of the family *Herpesviridae* exhibit 2 distinct phases in their biological cycle: lytic replication and latency. While lytic replication is associated with production of viral particles, latency consists in the maintenance of the viral genome as a nonintegrated episome and the expression of very few viral genes and microRNAs. Upon reactivation, latency is replaced by lytic replication. Even if latency has not been demonstrated conclusively in members of the *Alloherpesviridae* family, an increasing number of evidences support the existence of latent phase. These evidences related to CyHV-3 can be summarized as follows. (*i*) CyHV-3 DNA has been detected in the brain of fish that survived primary infection and showing no clinical signs at 64 dpi [42], and even 1 year post-infection [77]. (*ii*) CyHV-3 persisted in the wild population of common carp for at least 2 years after initial outbreak [46]. (*iii*) Finally, St-Hilaire *et al.* described, in fish that survived the primary infection, the induction of CyHV-3 reactivation by temperature stress several months after the initial exposure to the virus [43]. Increased level of viral DNA in gills without the appearance of disease symptoms has been detected after stress induced by netting fish that survived the primary infection and were kept at 20°C for 81 dpi [78]. Recent studies demonstrated that virus may become latent in white blood cells and other tissues, remains at very low copy numbers and can be reactivated by temperature stress [76, 79, 80]. To date, the temperature-dependent reactivation of the disease which resulted in mortality of naïve cohabitant fish has been described after transferring the fish maintained at a low temperature to the higher, permissive temperature [43, 79]. These observations suggest that the temperature of the water could regulate the switch between latency and lytic replication and vice versa allowing the virus to persist in the host population throughout the seasons even when the temperature is non-permissive.

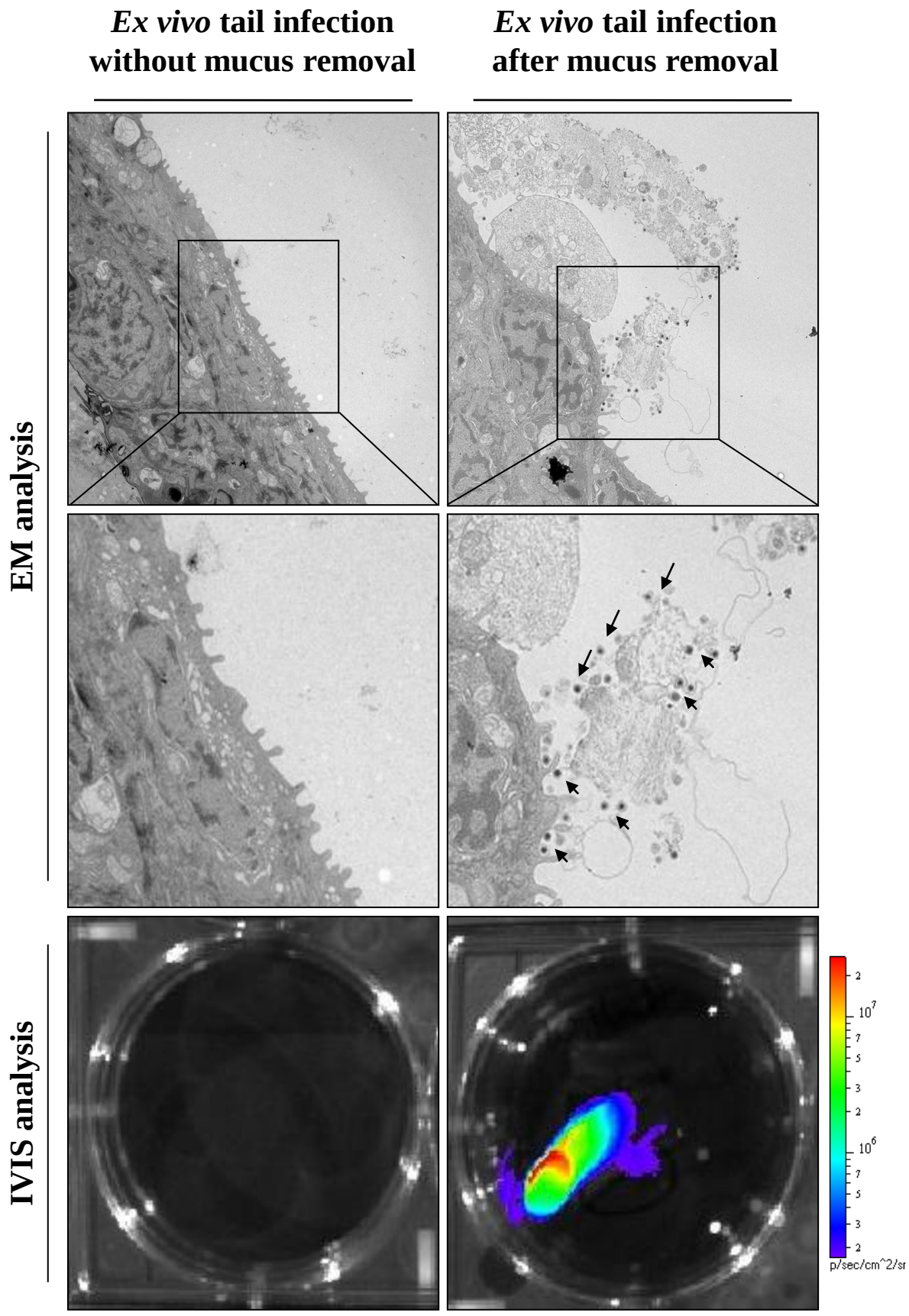


Figure 6. Effect of skin mucus removal on CyHV-3 binding to carp epidermal cells. Tail fin ventral lobes of carp were mock-treated or treated by rubbing with a soft tissue paper to remove epidermal mucus. Immediately after skin treatment, tail fin explants were harvested and inoculated ex vivo with a CyHV-3 recombinant strain expressing luciferase as a reporter gene (10^6 PFU/mL of culture medium for 2 h). At the end of the 2 h inoculation period, a fragment of the fin was collected and processed for electron microscopy examination (EM analysis). The arrows indicate CyHV-3 particles bound to cells or cell debris. Twenty-four hours post-inoculation, duplicate tail explant cultures were analyzed by bioluminescence imaging (lower panels). Reproduced with permission from Raj et al. [75].

3.4 Transmission

To date, no evidence of CyHV-3 vertical transmission has been reported. Horizontal transmission of CyHV-3 occurs either by direct transmission (fish to fish) or vector based transmissions. Direct transmission can be by skin to skin contact of infected carp or cyprinid and non-cyprinid fish species that can carry CyHV-3 asymptomatically [64, 68] against naïve carp; or by cannibalistic and necrophagous behaviors of the carp [22, 57]. Several potential vectors could be involved in the vector based transmission of CyHV-3. Such vectors include fish droppings [81], plankton [82], aquatic invertebrates feeding by water filtration [54], piscivorous birds which could transfer the disease by moving sick fish from one pond to another [83], and finally the water being the major abiotic vector. Secretion of viral particles into the water either through shedding or together with sloughed epithelial cells has been documented [59]. Furthermore, the infectivity of CyHV-3 in water was shown to be conserved for at least 4 h [58], even if longer period could be observed depending on water composition (chemical and microbial) [84]. For example, the infectivity of CyHV-3 was drastically reduced after 3 days in environmental water, although it remained quite stable for more than 7 days in sterilized water [84].

3.5 Diagnosis

Various CyHV-3 diagnostic methods have been developed. They are based on the detection of infectious particles, viral DNA, transcripts, or antigens. Virus isolation from infected fish tissues in cell culture was the first method developed [9, 11]. Although cell culture isolation is not as sensitive as PCR-based methods, it is the only technique able to detect infectious particles. Recently, Dong *et al.* isolated for the first time CyHV-3 virus from diseased koi in mainland China using a newly developed cell line from caudal fin of koi [34]. A complete set of molecular techniques for detection of viral DNA fragments has been developed, such as DNA hybridization, PCR, nested PCR, one-tube semi-nested PCR, semi-quantitative PCR, real-time TaqMan PCR, and loop-mediated isothermal amplification [22]. CyHV-3 genome can also be detected and quantified in environmental water by real-time TaqMan PCR after viral concentration [85]. Recently, a mRNA-specific RT-PCR assay for detection of replicating CyHV-3 in infected fish tissues and cell cultures has been described [86]. ELISA tests have been developed to detect specific anti-CyHV-3 antibodies in carp serum [22]. CyHV-3 has been also detected in tissues and touch imprints of organs from infected fish by immunohistochemistry and immunofluorescence assays, respectively [59]. Monoclonal antibodies against CyHV-3 ORF68 have been produced. They were proved to detect specifically CyHV-3 without cross-reaction against CyHV-1 and CyHV-2 [87]. Finally, a CyHV-3-detection kit (The FASTest® Koi HV kit) that allows the detection of CyHV-3 in gill swabs in just 15 min has been developed [88].

3.6 Vaccination

Soon after the identification of CyHV-3 as the causative agent of koi herpesvirus disease (KHVD), an original protocol was developed to induce a protective adaptive immune response in carp [11]. This approach exploited the fact that CyHV-3 induces fatal infections only when temperature is between 18°C and 28°C. According to this protocol, healthy fingerlings are exposed to the virus by cohabitation with sick fish for 3–5 days at permissive temperature (22°C-23°C). After that the fish are transferred to ponds for 25–30 days at non-permissive water temperature (≈30°C). Despite its ingenuity, this protocol has several disadvantages. (*i*) Fish that are "vaccinated" with this protocol become latently infected by a virulent strain and are therefore likely to represent a potential source of CyHV-3 outbreaks if they later cohabitate with naïve carp. (*ii*) The increase of water temperature to non-permissive is costly and correlated with increasing susceptibility of the fish to secondary infection. (*iii*) Finally, after this procedure only 60% of immunized fish proved to be resistant to a CyHV-3 challenge performed by cohabitation with infected fish [11].

Attenuated live vaccines appear to be the most appropriate for mass vaccination of carp. Live attenuated vaccine candidates have been produced by serial passages in cell culture of a pathogenic strain. A vaccine strain candidate was further attenuated by UV irradiation in order to increase random mutations throughout the genome [11, 89]. Currently, a live attenuated vaccine developed using this approach has been manufactured by KoVax Ltd. (Jerusalem, Israel) and is available for immersion vaccination of common and koi carp in Israel [90]. Protection against CyHV-3 is associated with elevation of specific antibodies against the virus [11, 89]. However, the duration of the protection conferred by the vaccine has not been established [90]. This vaccine has two major additional disadvantages: (*i*) the determinism of the attenuation is unknown; and consequently, reversions to a pathogenic phenotype cannot be excluded; (*ii*) the attenuated strain retains residual virulence that could be lethal for a portion of the vaccinated fish [91], particularly for small/young fish.

An inactivated vaccine candidate was also described by Yasumoto *et al.* [92]. It consists of formalin-inactivated CyHV-3 trapped within a liposomal compartment. This vaccine can be used for oral immunization in fish food. Protection efficacy for carp was 70% [92].

4. Host-pathogen interactions

4.1 Genetic resistance of carp strains to CyHV-3

Genetic differences in resistance to CyHV-3 have been described among different carp strains and crossbreeds. Independent research groups demonstrated that resistance to CyHV-3 can be significantly increased by crossing of domesticated carp strains with wild carp strains. Shapira *et al.* reported that the most resistant carp crossbreed in their study (60% of survival) was that between the domesticated carp strain Dor-70 and the wild carp strain Sassan [93]. In comparison the survival rate

of domesticated carp strains Našice and Dor-70 as well as their crossbreed was much lower (8%, 27% and 17.7%, respectively) [93]. Recently, Piačková *et al.* demonstrated that most of Czech strains and crossbreeds which are genetically related to wild Amur carp were significantly more resistant to CyHV-3 infection than strains with no relation to Amur carp [94]. Carp genetic resistance to CyHV-3 has been investigated using 96 carp families derived from diallelic crossing of two wild carp strains (Amur and Duna) and two domesticated Hungarian strains (Tat and HAKI 15) [95]. This study demonstrated that crossing with wild carp strains may result in higher resistance to CyHV-3. However, individual parents of the strains are also important since many of the families derived from the wild strains did not exhibit significantly higher resistance [95]. Recently, resistance to CyHV-3 has been also linked to the polymorphism of the MHC class II *B* genes [56] and carp IL-10 gene [96]. These findings support the hypothesis that the outcome of the disease can be controlled in some extent by genetic factors of the host, and consequently, that selection of resistant carp breeders is one of potential ways to reduce the negative impact of CyHV-3 on carp aquaculture.

4.2 Immune response of carp against CyHV-3

Knowledge on the immune mechanisms and immunological traits that can correlate with disease resistance in fish as well as on the immune evasion mechanisms expressed by CyHV-3, is essential for the development of prophylactic strategies (such as vaccination) as well as for the development of more resistant strains by the use of molecular marker assisted selection. The information related to these topics are summarized in this section.

Perelberg *et al.* studied the kinetic of anti-CyHV-3 antibody expression in the serum of carp infected at different temperatures [91]. In fish that were infected and maintained at 24°C, antibody titers began to rise at 10 dpi and reached a peak around 20-40 dpi. It was shown that protection against CyHV-3 is proportional to the titer of specific antibodies produced during the primary infection. The level of antibodies decreased in the absence of antigenic re-exposure. At 280 dpi, the titer of anti-CyHV-3 antibodies of infected fish was only slightly higher or comparable to that of unexposed fish. Nevertheless, immunized fish, even those in which antibodies were no longer detectable were resistant to a lethal challenge; possibly because of the subsequent rapid response of B and T memory cells to antigen re-stimulation [91].

Recently, a transcriptomic study uncovered the wide array of immune-related genes involved in the anti-CyHV-3 immune response of carp [97]. The response of two carp lines with different resistance to CyHV-3 has been studied using DNA microarray and real-time PCR. Significantly higher expression of several immune-related genes including number of those which are involved in pathogen recognition, complement activation, MHC class I-restricted antigen presentation and development of adaptive mucosal immunity was noted in more resistant carp line. Further real-time PCR based analyses provided evidence for higher activation of $CD8^+$ T cells in the more resistant carp line. Thus,

differences in resistance to CyHV-3 can be correlated with differentially expressed immune-related genes [97].

The anti-CyHV-3 immune response has been studied in the skin and the intestine of common carp [60, 98]. These studies revealed an up-regulation of pro-inflamatory cytokine IL-1β, the inducible nitric oxide synthase (iNOS) and activation of interferon class I pathways [60, 98]. In skin, CyHV-3 infection leads to down-regulation of genes encoding several important components of the skin-mucosal barrier, including antimicrobial peptides (beta defensing 1 and 2), mucin 5B, and tight junction proteins (claudin 23 and 30). This probably contributes to changes in the skin bacterial flora and subsequent development of secondary bacterial infections [60]. Raj *et al.* demonstrated that skin mucus also acts as an innate immune barrier and inhibits CyHV-3 binding to epidermal cells at least partially by neutralisation of viral infectivity [75]. *In vitro* study demonstrated that CyHV-3 inhibits activity of stimulated macrophages and proliferative response of lymphocytes and that this effect is temperature dependent [99].

4.2.1 Interferon type I response

Interferons (IFNs) are secreted mediators that play essential roles in the innate immune response against viruses. *In vitro* studies demonstrated that CyHV-3 inhibits IFN type I secretion in CCB cells [100]. Poly I:C stimulation of CCB cells prior to CyHV-3 infection activated the IFN type I response and reduced CyHV-3 spreading in the cell culture [100]. *In vivo* studies showed that CyHV-3 induced a systemic IFN type I response in carp skin and intestine and that the magnitude of IFN type I expression is correlated with the virus load [60, 98].

Recently, Tomé *et al.* demonstrated that CyHV-3 ORF112 encodes a new Z-domain family protein which *in vitro* showed structural and functional properties similar to the poxvirus E3L inhibitor of interferon response [101]. This suggested that CyHV-3 may use similar mechanisms to inhibit interferon response as poxviruses. However, the potential function of ORF112 in virus pathogenesis *in vivo* has not been studied yet.

4.2.2 The role of CyHV-3 IL-10 homologue

CyHV-3 ORF134 encodes a viral homologue of cellular IL-10 [18]. Its expression product is a 179 amino acid protein [102]. Common carp IL-10 and CyHV-3 IL-10 exhibit 26.9% identity (67.3% similarity) over a 156 amino acid overlap [103]. Transcriptomic analyses revealed that ORF134 is expressed as a spliced gene belonging to the early [37] or early-late class [31]. Proteomic analyses of CyHV-3 infected cell supernatant demonstrated that ORF134 expression product is one of the most abundant proteins of the CyHV-3 secretome [31]. In CyHV-3 infected carp, ORF134 is highly expressed during acute and reactivation phase, while is expressed on a low level during low-temperature induced persistent phase [102]. *In vivo* study using a zebrafish embryo model

suggested that CyHV-3 ORF134 encodes a functional IL-10 homologue [102]. Injection of mRNA encoding CyHV-3 IL-10 into zebrafish embryos increased the number of lysozyme-positive cells to a similar degree as observed with zebrafish IL-10 [102]. Moreover, down-regulation of the IL-10 receptor long chain (IL-10R1) using a specific morpholino abrogated the increase of the number of lysozyme-positive cells after co-injection with either CyHV-3 IL-10 mRNA or zebrafish IL-10 mRNA, indicating that it functions via the IL-10 receptor [102].

Recently, a CyHV-3 strain deleted for ORF134 and a derived revertant strain were produced using BAC cloning technologies [31]. The recombinant ORF134 deleted strain replicated *in vitro* comparably to the parental and the revertant strains. Infection of fish by immersion in water containing the virus induced comparable mortality for the three virus genotypes tested (wild type, deleted and revertant). Quantification of viral DNA by real time TaqMan PCR and analysis of carp cytokines expression by RT-qPCR at different times post-infection did not reveal any significant difference between the groups of fish infected with the three virus genotypes. Moreover, histological examination of infected fish did not reveal significant differences between fish infected with the three genotypes. Altogether, these results demonstrated that the IL-10 homologue encoded by CyHV-3 is essential neither for viral replication *in vitro* nor for virulence *in vivo* [31].

5. Conclusions

Since its first description in the late 1990s, CyHV-3 rapidly spread to different continents (Europa, Asia, North America, Africa) causing severe financial losses in the common carp and koi culture industries worldwide. In addition to its negative economical and societal impacts, CyHV-3 has also a negative environmental impact by affecting wild populations of carp. These reasons explain why CyHV-3 became rapidly a subject for applied science and is now listed as a notifiable disease by the OIE. In addition to its economic importance, recent studies demonstrated that CyHV-3 is also a very attractive and original subject of fundamental research: (*i*) it is phylogenetically distant from the vast majority of herpesviruses that have been studied so far (the latter belong to the family *Herpesviridae*), thereby providing an original field of research. (*ii*) It can be studied in laboratories by infection of its natural host (homologous virus-host model). (*iii*) The sequence of its genome published recently revealed a fascinating virus with unique properties in the *Herpesvirales*, such as an extremely large genome (295 Kb), a high number of genes which are not homologous to known viral sequences, and genes that are normally found exclusively in the *Poxviridae* [18]. (*iv*) Importantly, the CyHV-3 genome revealed several genes encoding proteins potentially involved in immune evasion mechanisms. (v) Last but not least, the outcome of CyHV-3 infection is highly dependent on the temperature of the water in which the carp are maintained.

6. Competing interests

Dr Vanderplasschen's group is developing vaccine candidates against CyHV-3 as well as reagents to diagnose the disease.

7. Author's contributions

KR, PO, and AV contributed to the design of the structure of the manuscript. KR and AV drafted the manuscript. KR, PO, MB, MR, AR, CV, JJ-R, and AV performed the overview of the literature on CyHV-3, read and approved the final manuscript.

8. Acknowledgments

PO is a research fellow of the Chinese Scholarship Council. This work was supported by a grant from the University of Liège (Postdoc-IN program) and by grants of the "Fonds National Belge de la Recherche Scientifique" (FNRS) (R.FNRS.2165, -2697). KR and AV are members of the BELVIR consortium (IAP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).

9. References

1. Balon EK: **Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers.** *Aquaculture* 1995, **129:**3–48.
2. Statistics and Information Service of the Fisheries and Aquaculture Department: *Fishery and Aquaculture Statistics 2010.* Rome: FAO; 2012.
3. Balon EK: **The oldest domesticated fishes, and the consequences of an epigenetic dichotomy in fish culture.** *J Ichthyol Aquat Biol* 2006, **11:**47–86.
4. Magel GD, Tyring S: *Herpesviridae- a look into this unique family of viruses.* Shanghai: inTech; 2012.
5. Wolf K, Darlingtron RW: **Channel catfish virus: a new herpesvirus of ictalurid fish.** *J Virol* 1971, **8:**525–533.
6. Hedrick RP, McDowell TS, Groff JM, Yun S, Wingfield WH: **Isolation of an epitheliotropic herpesvirus from white sturgeon *Acipenser transmontanus*.** *Dis Aquat Organ* 1991, **11:**49–56.
7. Haenen OLM, Dijkstra SG, Van Tulden PW, Davidse A, Van Nieuwstadt AP, Wagenaar F, Wellenberg GJ: **Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in the Netherlands since 1996.** *Bull Eur Assoc Fish Pathol* 2002, **22:**247–257.
8. Bretzinger A, Fischer-Scherl T, Oumouna M, Hoffmann R, Truyen U: **Mass mortalities in Koi carp, *Cyprinus carpio*, associated with gill and skin disease.** *Bull Eur Assoc Fish Pathol* 1999, **19:**182–185.
9. Hedrick RP, Gilad O, Yun S, Spangenberg J, Marty GD, Nordhausen RW, Kebus M, Bercovier H, Eldar A: **A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp.** *J Aquat Anim Health* 2000, **12:**44–57.
10. Haenen OLM, Way K, Bergmann SM, Ariel E: **The emergence of koi herpesvirus and its significance to European aquaculture.** *Bull Eur Assoc Fish Pathol* 2004, **24:**293–307.
11. Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, Bejerano I, Steinitz M, Kotler M: **Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*.** *Vaccine* 2003, **21:**4677–4684.
12. Waltzek TB, Kelley GO, Stone DM, Way K, Hanson L, Fukuda H, Hirono I, Aoki T, Davison AJ, Hedrick RP: **Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*.** *J Gen Virol* 2005, **86:**1659–1667.
13. Davison AJ, Kurobe T, Gatherer D, Cunningham C, Korf I, Fukuda H, Hedrick RP, Waltzek TB: **Comparative genomics of carp herpesviruses.** *J Virol* 2013, **87:**2908–2922.
14. Waltzek TB, Kelley GO, Alfaro ME, Kurobe T, Davison AJ, Hedrick RP: **Phylogenetic relationships in the family *Alloherpesviridae*.** *Dis Aquat Organ* 2009, **84:**179–194.

15. Miyazaki T, Kuzuya Y, Yasumoto S, Yasuda M, Kobayashi T: **Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV.** *Dis Aquat Organ* 2008, **80:**1–11.
16. Miwa S, Ito T, Sano M: **Morphogenesis of koi herpesvirus observed by electron microscopy.** *J Fish Dis* 2007, **30:**715–722.
17. Mettenleiter TC, Klupp BG, Granzow H: **Herpesvirus assembly: an update.** *Virus Res* 2009, **143:**222–234.
18. Aoki T, Hirono I, Kurokawa K, Fukuda H, Nahary R, Eldar A, Davison AJ, Waltzek TB, Bercovier H, Hedrick RP: **Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide.** *J Virol* 2007, **81:**5058–5065.
19. Costes B, Fournier G, Michel B, Delforge C, Raj VS, Dewals B, Gillet L, Drion P, Body A, Schynts F, Lieffrig F, Vanderplasschen A: **Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*.** *J Virol* 2008, **82:**4955–4964.
20. Michel B, Leroy B, Stalin Raj V, Lieffrig F, Mast J, Wattiez R, Vanderplasschen AF, Costes B: **The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions.** *J Gen Virol* 2010, **91:**452–462.
21. Van Beurden SJ, Bossers A, Voorbergen-Laarman MH, Haenen OL, Peters S, Abma-Henkens MH, Peeters BP, Rottier PJ, Engelsma MY: **Complete genome sequence and taxonomic position of anguillid herpesvirus 1.** *J Gen Virol* 2010, **91:**880–887.
22. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A: **Cyprinid herpesvirus 3.** *Emerg Infect Dis* 2010, **16:**1835–1843.
23. Bigarré L, Baud M, Cabon J, Antychowicz J, Bergmann SM, Engelsma M, Pozet F, Reichert M, Castric J: **Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR.** *J Virol Methods* 2009, **158:**51–57.
24. Kurita J, Yuasa K, Ito T, Sano M, Hedrick RP, Engelsma MY, Haenen OLM, Sunarto A, Kholidin EB, Chou H-Y, Tung M-C, de la Peña L, Lio-Po G, Tu C, Way K, Iida T: **Molecular epidemiology of koi herpesvirus.** *Fish Pathol* 2009, **44:**59–66.
25. Sunarto A, McColl KA, Crane MS, Sumiati T, Hyatt AD, Barnes AC, Walker PJ: **Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage.** *J Fish Dis* 2011, **34:**87–101.
26. Avarre JC, Madeira JP, Santika A, Zainun Z, Baud M, Cabon J, Caruso D, Castric J, Bigarré L, Engelsma M, Maskur M: **Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable number of tandem repeats analysis.** *J Virol Methods* 2011, **173:**320–327.
27. Dong C, Li X, Weng S, Xie S, He J: **Emergence of fatal European genotype CyHV-3/KHV in mainland China.** *Vet Microbiol* 2013, **162:**239–244.

28. Han JE, Kim JH, Renault T, Choresca Jr. Shin SP, Jun JW, Park SC: **Identifying the viral genes encoding envelope glycoproteins for differentiation of Cyprinid herpesvirus 3 isolates.** *Viruses* 2013, **5:**568–576.

29. Rosenkranz D, Klupp BG, Teifke JP, Granzow H, Fichtner D, Mettenleiter TC, Fuchs W: **Identification of envelope protein pORF81 of koi herpesvirus.** *J Gen Virol* 2008, **89:**896–900.

30. Gotesman M, Soliman H, El-Matbouli M: **Antibody screening identifies 78 putative host proteins involved in Cyprinid herpesvirus 3 infection or propagation in common carp, *Cyprinus carpio* L.** *J Fish Dis* 2013, **36:**721–733.

31. Ouyang P, Rakus K, Boutier M, Reschner A, Leroy B, Ronsmans M, Fournier G, Scohy S, Costes B, Wattiez R, Vanderplasschen A: **The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*.** *Vet Res* 2013, **44:**53.

32. Neukirch M, Böttcher K, Bunnajirakul S: **Isolation of a virus from koi with altered gills.** *Bull Eur Assoc Fish Pathol* 1999, **19:**221–224.

33. Neukirch M, Kunz U: **Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality.** *Bull Eur Assoc Fish Pathol* 2001, **21:**125–135.

34. Dong C, Weng S, Li W, Li X, Yi Y, Liang Q, He J: **Characterization of a new cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid herpesvirus 3 in China.** *Virus Res* 2011, **161:**140–149.

35. Davidovich M, Dishon A, Ilouze M, Kotler M: **Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3.** *Arch Virol* 2007, **152:**1541–1546.

36. Oh MJ, Jung SJ, Choi TJ, Kim HR, Rajendran KV, Kim YJ, Park MA, Chun SK: **A viral disease occurring in cultured carp *Cyprinus carpio* in Korea.** *Fish Pathol* 2001, **36:**147–151.

37. Ilouze M, Dishon A, Kotler M: **Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes.** *Virus Res* 2012, **169:**98–106.

38. Fuchs W, Fichtner D, Bergmann SM, Mettenleiter TC: **Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism.** *Arch Virol* 2011, **156:**1059–1063.

39. Le Morvan C, Troutaud D, Deschaux P: **Differential effects of temperature on specific and nonspecific immune defences in fish.** *J Exp Biol* 1998, **201:**165–168.

40. Dishon A, Davidovich M, Ilouze M, Kotler M: **Persistence of cyprinid herpesvirus 3 in infected cultured carp cells.** *J Virol* 2007, **81:**4828–4836.

41. Ilouze M, Dishon A, Kotler M: **Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature.** *Virus Res* 2012, **169:**289–295.

42. Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP: **Concentrations of a *Koi herpesvirus* (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR.** *Dis Aquat Organ* 2004, **60:**179–187.

43. St-Hilaire S, Beevers N, Way K, Le Deuff RM, Martin P, Joiner C: **Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*.** *Dis Aquat Organ* 2005, **67:**15–23.

44. St-Hilaire S, Beevers N, Joiner C, Hedrick RP, Way K: **Antibody response of two populations of common carp, *Cyprinus carpio* L., exposed to koi herpesvirus.** *J Fish Dis* 2009, **32:**311–320.

45. Yuasa K, Ito T, Sano M: **Effect of water temperature on mortality and virus shedding in carp experimentally infected with koi herpesvirus.** *Fish Pathol* 2008, **43:**83–85.

46. Uchii K, Matsui K, Iida T, Kawabata Z: **Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, *Cyprinus carpio* L.** *J Fish Dis* 2009, **32:**857–864.

47. Denham KL: **Koi herpesvirus in wild fish.** *Vet Rec* 2003, **153:**507.

48. Terhune JS, Grizzle JM, Hayden K, McClenahan SD, Lamprecht SD, White MG: **First report of koi herpesvirus in wild common carp in the western hemisphere.** *Fish Health Newsletter* 2004, **32:**8–9.

49. Grimmett SG, Warg JV, Getchell RG, Johnson DJ, Bowser PR: **An unusual koi herpesvirus associated with a mortality event of common carp *Cyprinus carpio* in New York State, USA.** *J Wildl Dis* 2006, **42:**658–662.

50. Garver KA, Al-Hussinee L, Hawley LM, Schroeder T, Edes S, LePage V, Contador E, Russell S, Lord S, Stevenson RM, Souter B, Wright E, Lumsden JS: **Mass mortality associated with koi herpesvirus in wild common carp in Canada.** *J Wildl Dis* 2010, **46:**1242–1251.

51. Minamoto T, Honjo MN, Uchii K, Yamanaka H, Suzuki AA, Kohmatsu Y, Iida T, Kawabata Z: **Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak.** *Vet Microbiol* 2009, **135:**261–266.

52. Minamoto T, Honjo MN, Yamanaka H, Uchii K, Kawabata Z: **Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan.** *Res Vet Sci* 2012, **93:**508–514.

53. Honjo MN, Minamoto T, Kawabata Z: **Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds.** *Vet Microbiol* 2012, **155:**183–190.

54. Kielpinski M, Kempter J, Panicz R, Sadowski J, Schütze H, Ohlemeyer S, Bergmann SM: **Detection of KHV in freshwater mussels and Crustaceans from ponds with KHV history in Common Carp (*Cyprinus carpio*).** *Isr J Aquac Bamidgeh* 2010, **62:**28–37.

55. Bergmann SM, Sadowski J, Kielpinski M, Bartlomiejczyk M, Fichtner D, Riebe R, Lenk M, Kempter J: **Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD).** *J Fish Dis* 2010, **33:**267–272.

56. Rakus KL, Wiegertjes GF, Adamek M, Siwicki AK, Lepa A, Irnazarow I: **Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism.** *Fish Shellfish Immunol* 2009, **26:**737–743.

57. Fournier G, Boutier M, Stalin Raj V, Mast J, Parmentier E, Vanderwalle P, Peeters D, Lieffrig F, Farnir F, Gillet L, Vanderplasschen A: **Feeding *Cyprinus carpio* with infectious materials**

mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Vet Res* 2012, **43:**6.

58. Perelberg A, Smirnov M, Hutoran M, Diamant A, Bejerano Y, Kotler M: **Epidemiological description of a new viral disease afflicting cultured *Cyprinus Carpio* in Israel.** *Isr J Aquac Bamidgeh* 2003, **55:**5–12.
59. Pikarsky E, Ronen A, Abramowitz J, Levavi-Sivan B, Hutoran M, Shapira Y, Steinitz M, Perelberg A, Soffer D, Kotler M: **Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus.** *J Virol* 2004, **78:**9544–9551.
60. Adamek M, Syakuri H, Harris S, Rakus KL, Brogden G, Matras M, Irnazarow I, Steinhagen D: **Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.).** *Vet Microbiol* 2013, **162:**456–470.
61. Hedrick RP, Waltzek TB, McDowell TS: **Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpes virus-3.** *J Aquat Anim Health* 2006, **18:**26–34.
62. Bergmann SM, Schütze H, Fischer U, Fichtner D, Riechardt M, Meyer K, Schrudde D, Kempter J: **Detection KHV genome in apparently health fish.** *Bull Eur Assoc Fish Pathol* 2009, **29:**145-152.
63. Kempter J, Bergmann SM: **Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland.** *Aquaculture* 2007, **272:**S275–S275.
64. Kempter J, Kielpinski M, Panicz R, Sadowski J, Mysłowski B, Bergmann SM: **Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp.** *Bull Eur Assoc Fish Pathol* 2012, **32:**212–219.
65. Fabian M, Baumer A, Steinhagen D: **Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds?** *J Fish Dis* 2012, **36:**505–514.
66. Kempter J, Sadowski J, Schütze H, Fischer U, Dauber M, Fichtner D, Panicz R, Bergmann SM: **Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones?** *Acta Ichthyol Piscat* 2009, **39:**119–126.
67. El-Matbouli M, Saleh M, Soliman H: **Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (*Cyprinus carpio koi*).** *Vet Rec* 2007, **161:**792–793.
68. El-Matbouli M, Soliman H: **Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naive common carp by cohabitation.** *Res Vet Sci* 2011, **90:**536–539.
69. Bergmann SM, Lutze P, Schütze H, Fischer U, Dauber M, Fichtner D, Kempter J: **Goldfish (*Carassius auratus auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD).** *Bull Eur Assoc Fish Pathol* 2010, **30:**74–84.
70. Radosavljević V, Jeremić S, Ćirković M, Lako B, Milićević V, Potkonjak A, Nikolin V: **Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers.** *Acta Vet* 2012, **62:**675–681.

71. OIE (World Organisation for Animal Health): *Report of the meeting of the OIE aquatic animal health standards commission.* Paris; 2012. http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Aquatic_Commission/A_AAC_March_2012.pdf.

72. Ito T, Sano M, Kurita J, Yuasa K, Iida T: **Carp larvae are not susceptible to Koi Herpesvirus.** *Fish Pathol* 2007, **42:**107–109.

73. Ronsmans M, Rougeot C, Melard C, Vanderplasschen A: *Susceptibility of Cyprinus carpio to Cyprinid herpesvirus-3 at the early stages of life.* The Netherlands: Fish Immunology Workshop, Wageningen; 2012 April.

74. Costes B, Raj VS, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Bremont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin.** *J Virol* 2009, **83:**2819–2830.

75. Raj VS, Fournier G, Rakus K, Ronsmans M, Ouyang P, Michel B, Delforges C, Costes B, Farnir F, Leroy B, Wattiez R, Melard C, Mast J, Lieffrig F, Vanderplasschen A: **Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells.** *Vet Res* 2011, **42:**92.

76. Eide K, Miller-Morgan T, Heidel J, Bildfell R, Jin L: **Results of total DNA measurement in koi tissue by Koi Herpesvirus real-time PCR.** *J Virol Methods* 2011, **172:**81–84.

77. Yuasa K, Sano M: **Koi herpesvirus: status of outbreaks, diagnosis, surveillance, and research.** *Isr J Aquac Bamidgeh* 2009, **61:**169–179.

78. Bergmann SM, Kempter J: **Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods.** *Bull Eur Assoc Fish Pathol* 2011, **31:**92–100.

79. Eide KE, Miller-Morgan T, Heidel JR, Kent ML, Bildfell RJ, LaPatra S, Watson G, Jin L: **Investigation of koi herpesvirus latency in koi.** *J Virol* 2011, **85:**4954–4962.

80. Xu JR, Bently J, Beck L, Reed A, Miller-Morgan T, Heidel JR, Kent ML, Rockey DD, Jin L: **Analysis of koi herpesvirus latency in wild common carp and ornamental koi in Oregon, USA.** *J Virol Methods* 2013, **187:**372–379.

81. Dishon A, Perelberg A, Bishara-Shieban J, Ilouze M, Davidovich M, Werker S, Kotler M: **Detection of carp interstitial nephritis and gill necrosis virus in fish droppings.** *Appl Environ Microbiol* 2005, **71:**7285–7291.

82. Minamoto T, Honjo MN, Yamanaka H, Tanaka N, Itayama T, Kawabata Z: **Detection of cyprinid herpesvirus-3 DNA in lake plankton.** *Res Vet Sci* 2011, **90:**530–532.

83. Ilouze M, Davidovich M, Diamant A, Kotler M, Dishon A: **The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: A review.** *Ecol Res* 2011, **26:**885–892.

84. Shimizu T, Yoshida N, Kasai H, Yoshimizu M: **Survival of koi herpesvirus (KHV) in environmental water.** *Fish Pathol* 2006, **41:**153–157.

85. Honjo MN, Minamoto T, Matsui K, Uchii K, Yamanaka H, Suzuki AA, Kohmatsu Y, Iida T, Kawabata Z: **Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus.** *Appl Environ Microbiol* 2010, **76:**161–168.

86. Yuasa K, Kurita J, Kawana M, Kiryu I, Oseko N, Sano M: **Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage.** *Dis Aquat Organ* 2012, **100:**11–18.

87. Aoki T, Takano T, Unajak S, Takagi M, Kim YR, Park SB, Kondo H, Hirono I, Saito-Taki T, Hikima J, Jung TS: **Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus.** *Comp Immunol Microbiol Infect Dis* 2011, **34:**209–216.

88. Vrancken R, Boutier M, Ronsmans M, Reschner A, Leclipteux T, Lieffrig F, Collard A, Mélard C, Wera S, Neyts J, Goris N, Vanderplasschen A: **Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs.** *J Virol Methods* 2013, **193:**679–682.

89. Perelberg A, Ronen A, Hutoran M, Smith Y, Kotler M: **Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine.** *Vaccine* 2005, **23:**3396–3403.

90. Israel: KoVax Ltd. http://www.kovax.co.il/products/kv3-vaccine-against-khv/.

91. Perelberg A, Ilouze M, Kotler M, Steinitz M: **Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3).** *Vaccine* 2008, **26:**3750–3756.

92. Yasumoto S, Kuzuya Y, Yasuda M, Yoshimura T, Miyazaki T: **Oral immunization of common carp with a liposome vaccine fusing Koi herpesvirus antigen.** *Fish Pathol* 2006, **41:**141–145.

93. Shapira Y, Magen Y, Zak T, Kotler M, Hulata G, Levavi-Sivan B: **Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds.** *Aquaculture* 2005, **245:**1–11.

94. Piackova V, Flajshans M, Pokorova D, Reschova S, Gela D, Cizek A, Vesely T: **Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV).** *J Fish Dis* 2013, **36:**75–80.

95. Dixon PF, Joiner CL, Way K, Reese RA, Jeney G, Jeney Z: **Comparison of the resistance of selected families of common carp, *Cyprinus carpio* L., to koi herpesvirus: preliminary study.** *J Fish Dis* 2009, **32:**1035–1039.

96. Kongchum P, Sandel E, Lutzky S, Hallerman EM, Hulata G, David L, Palti Y: **Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (*Cyprinus carpio*).** *Aquaculture* 2011, **315:**417–421.

97. Rakus KL, Irnazarow I, Adamek M, Palmeira L, Kawana Y, Hirono I, Kondo H, Matras M, Steinhagen D, Flasz B, Brogden G, Vanderplasschen A, Aoki T: **Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses.** *Dev Comp Immunol* 2012, **37:**65–76.

98. Syakuri H, Adamek M, Brogden G, Rakus KL, Matras M, Irnazarow I, Steinhagen D: **Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: Molecular**

identification and regulation of the mRNA expression of claudin encoding genes. *Fish Shellfish Immunol* 2013, **34:**305–314.

99. Siwicki A, Kazuń K, Kazuń B, Majewicz-Zbikowska E: **Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes.** *Arch Pol Fish* 2012, **20:**123–128.
100. Adamek M, Rakus KL, Chyb J, Brogden G, Huebner A, Irnazarow I, Steinhagen D: **Interferon type I responses to virus infections in carp cells: *In vitro* studies on Cyprinid herpesvirus 3 and *Rhabdovirus carpio* infections.** *Fish Shellfish Immunol* 2012, **33:**482–493.
101. Tomé AR, Kuś K, Correia S, Paulo LM, Zacarias S, de Rosa M, Figueiredo D, Parkhouse RM, Athanasiadis A: **Crystal structure of a poxvirus-like zalpha domain from cyprinid herpesvirus 3.** *J Virol* 2013, **87:**3998–4004.
102. Sunarto A, Liongue C, McColl KA, Adams MM, Bulach D, Crane MSJ, Schat KA, Slobedman B, Barnes AC, Ward AC, Walker PJ: **Koi herpesvirus encodes and expresses a functional interleukin-10.** *J Virol* 2012, **86:**11512–11520.
103. Van Beurden SJ, Forlenza M, Westphal AH, Wiegertjes GF, Haenen OL, Engelsma MY: **The alloherpesviral counterparts of interleukin 10 in European eel and common carp.** *Fish Shellfish Immunol* 2011, **31:**1211–1217.

Introduction

3rd chapter:

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

Accepted for publication to Journal of General Virology

Ping Ouyang[1, ϕ], Krzysztof Rakus[1, ϕ], Steven J. van Beurden[1], Adrie Westphal[2], Andrew J. Davison[3], Derek Gatherer[3,4] and Alain F. Vanderplasschen[1]*

[1]Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium
[2]Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen UR, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[3]MRC–University of Glasgow Centre for Virus Research, 8 Church Street, Glasgow G11 5JR, United Kingdom
[4]Division of Biomedical & Life Sciences, Lancaster University, Lancaster LA1 4YQ, United Kingdom

[ϕ] These authors contributed equally

* Corresponding author. E-mail: a.vdplasschen@ulg.ac.be. Phone: +32 4 366 42 64

Abstract

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.

Key words: Cellular Interleukin-10 (cIL-10), Viral Interleukin 10 (vIL-10), IL-10 orthologues, Biological activities

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).

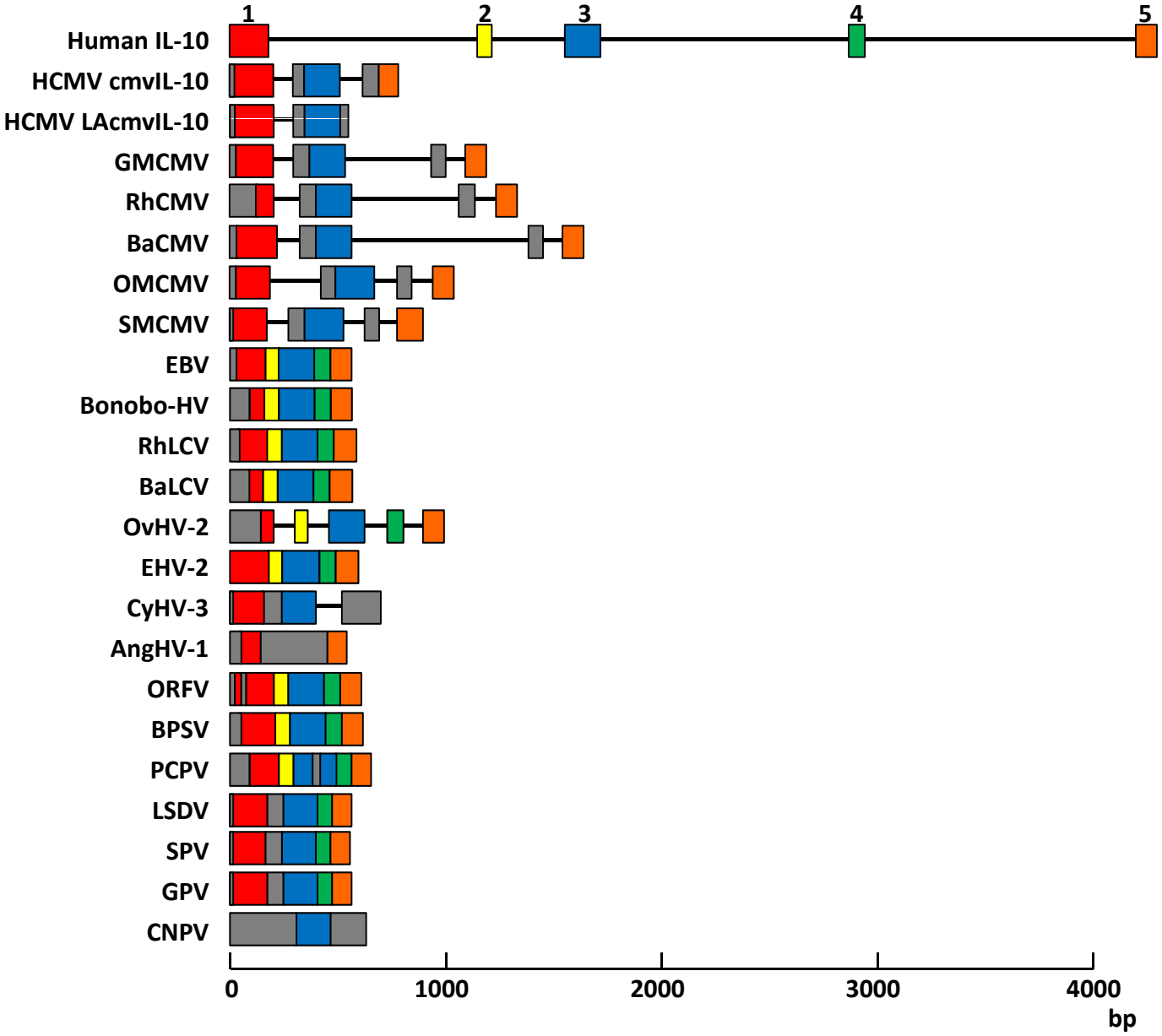


Figure 1. Schematic representation of the genomic intron/exon organization of human IL-10 (*H. sapiens*, Genbank ID: NP_000563) and vIL-10s encoded by the viruses listed in Table 1. Boxes and horizontal lines represent exons and introns, respectively. They are drawn to scale. The 5'- and 3'-UTRs of human 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.

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)

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.

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) (Figure. 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 Figure. 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

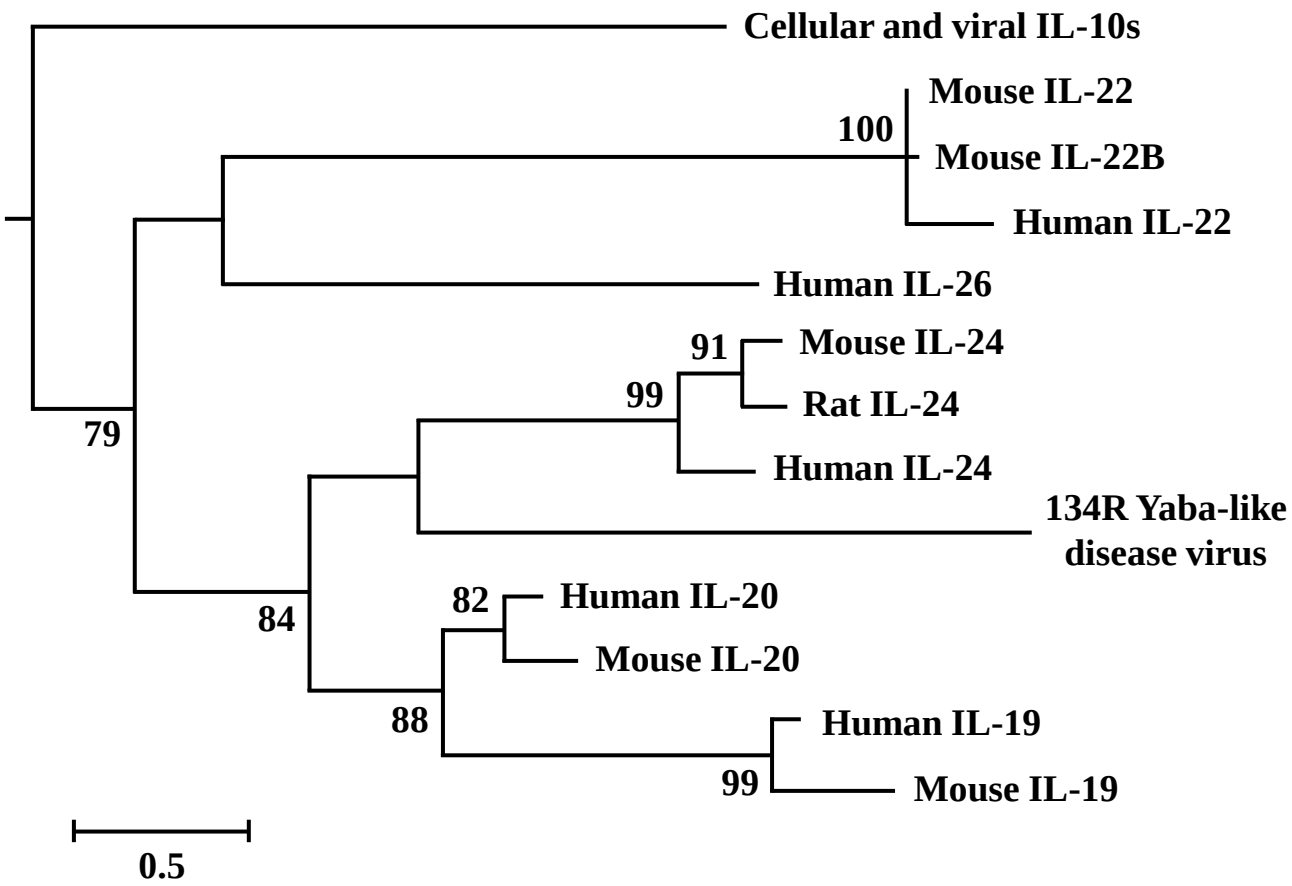


Figure 2. Maximum likelihood phylogenetic tree for cIL-10s, vIL-10s (listed in Table 1), the 134R protein encoded by Yaba-like disease virus, and selected members of the IL-20 family of cytokines. Sequences and methods used are described in supplementary file S1. The tree was build using MEGA (JTT+Γ 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.

bonobo herpesvirus (bonobo-HV) (The rational that lead to this conclusion is described in the supplementary material S2). Figure 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. Figure 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 (Figure 3). Based on Figure 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

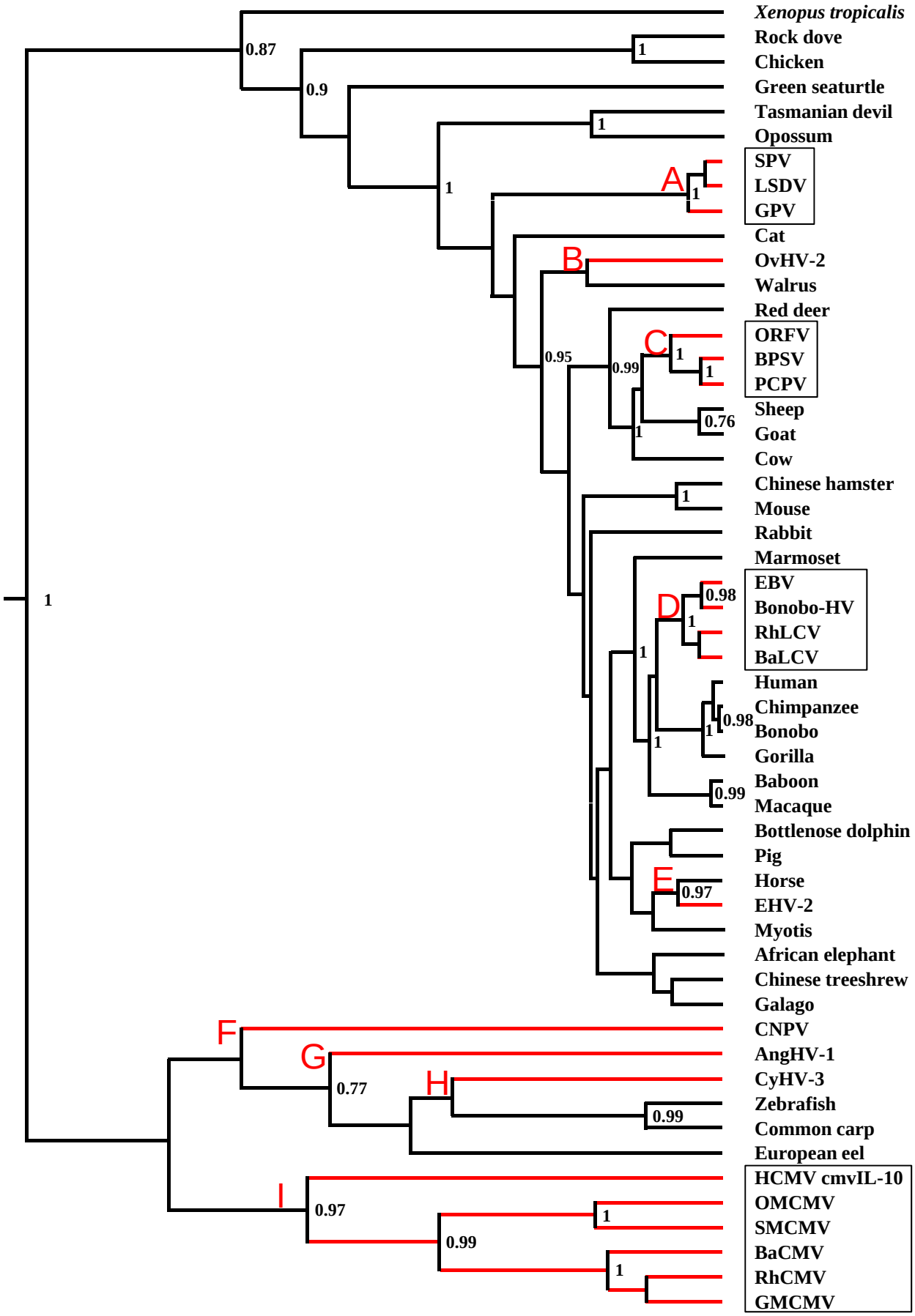


Figure 3. Bayesian consensus tree built using BEAST for cIL-10s and vIL-10s. Sequences and methods used are described in supplementary file S1. Figures at nodes are posterior probabilities (where >70%) of common ancestry. Branch lengths are arbitrary. Four positionally orthologous sets of vIL-10 are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

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 Figure 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;

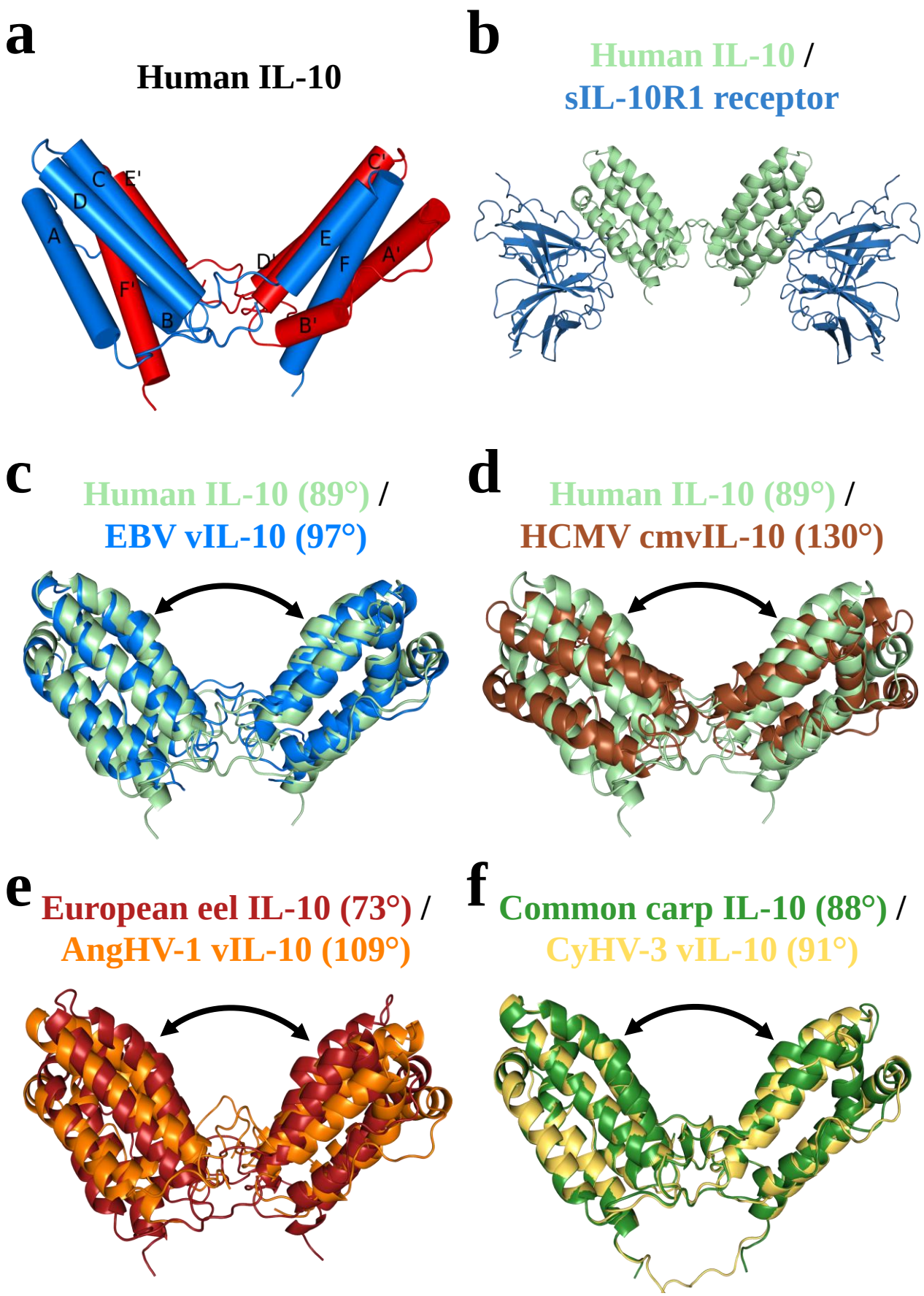


Figure 4. Structure of cIL-10s and selected vIL-10s. a) Crystal structure of human IL-10 from the IL-10/IL-10R1 complex (PDB ID: 1j7v (Josephson *et al.*, 2001)). IL-10 protomers are depicted with helices rendered as cylinders. Helices are labelled. b) Ribbon diagram of the 1:2 IL-10/sIL-10R1 complex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

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 (Figure 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 (Figure 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) (Figure 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) (Figure 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 BCRF1 deleted 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 signaling 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 IFN-γ 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 IFN-γ 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.

11. Acknowledgements

PO is a research fellow of the Chinese Scholarship Council. This work was supported by a grant from the University of Liège (Postdoc-IN program) and by grants of the 'Fonds National Belge de la Recherche Scientifique' (FNRS) (R.FNRS.2165, -2697). KR and AV are members of the BELVIR consortium (IAP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).

12. References

Allavena, P., Piemonti, L., Longoni, D., Bernasconi, S., Stoppacciaro, A., Ruco, L. & Mantovani, A. (1998). IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol* **28**, 359-369.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H. & other authors (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J Virol* **81**, 5058-5065.

Arrand, J. R., Rymo, L., Walsh, J. E., Björck, E., Lindahl, T. & Griffin, B. E. (1981). Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping restriction endonuclease fragments. *Nucleic Acids Res* **9**, 2999-3014.

Avdic, S., Cao, J. Z., Cheung, A. K., Abendroth, A. & Slobedman, B. (2011). Viral interleukin-10 expressed by human cytomegalovirus during the latent phase of infection modulates latently infected myeloid cell differentiation. *J Virol* **85**, 7465-7471.

Avdic, S., Cao, J. Z., McSharry, B. P., Clancy, L. E., Brown, R., Steain, M., Gottlieb, D. J., Abendroth, A. & Slobedman, B. (2013). Human cytomegalovirus interleukin-10 polarizes monocytes toward a deactivated M2c phenotype to repress host immune responses. *J Virol* **87**, 10273-10282.

Banchereau, J., Pascual, V. & O'Garra, A. (2012). From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat Immunol* **13**, 925-931.

Bartlett, N. W., Dumoutier, L., Renauld, J. C., Kotenko, S. V., McVey, C. E., Lee, H. J. & Smith, G. L. (2004). A new member of the interleukin 10-related cytokine family encoded by a poxvirus. *J Virol* **85**, 1401-1412.

Brady, M. T., MacDonald, A. J., Rowan, A. G. & Mills, K. H. G. (2003). Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur J Immunol* **33**, 3448-3457.

Bratke, K. A. & McLysaght, A. (2008). Identification of multiple independent horizontal gene transfers into poxviruses using a comparative genomics approach. *BMC Evol Biol* **8**, 67.

Brockman, M. A., Kwon, D. S., Tighe, D. P., Pavlik, D. F., Rosato, P. C., Sela, J., Porichis, F., Le Gall, S., Waring, M. T. & other authors (2009). IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. *Blood* **114**, 346-356.

Brodeur, N. D. & Spencer, J. V. (2010). Antibodies to human IL-10 neutralize ebvIL-10-mediated cytokine suppression but have no effect on cmvIL-10 activity. *Virus Res* **153**, 265-268.

Brunovskis, P. & Kung, H. J. (1995). Retrotransposition and herpesvirus evolution. *Virus genes* **11**, 259-270.

Cai, G., Kastelein, R. A. & Hunter, C. A. (1999). IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur J Immunol* **29**, 2658-2665.

Carson, W. E., Lindemann, M. J., Baiocchi, R., Linett, M., Tan, J. C., Chou, C.-C., Narula, S. & Caligiuri, M. (1995). The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood* **85**, 3577-3585.

Cassatella, M. A., Meda, L., Bonora, S., Ceska, M. & Constantin, G. (1993). Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. *J Exp Med* **178**, 2207-2211.

Chan, A., Baird, M., Mercer, A. A. & Fleming, S. B. (2006). Maturation and function of human dendritic cells are inhibited by orf virus-encoded interleukin-10. *J Gen Virol* **87**, 3177-3181.

Chang, W. L. & Barry, P. A. (2010). Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity. *Proc Natl Acad Sci U S A* **107**, 22647-22652.

Chang, W. L. W., Baumgarth, N., Yu, D. & Barry, P. A. (2004). Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J Virol* **78**, 8720-8731.

Chang, W. L., Barry, P. A., Szubin, R., Wang, D. & Baumgarth, N. (2009). Human cytomegalovirus suppresses type I interferon secretion by plasmacytoid dendritic cells through its interleukin 10 homolog. *Virology* **390**, 330-337.

Cheeran, M. C. J., Hu, S., Sheng, W. S., Peterson, P. K. & Lokensgard, J. R. (2003). CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10. *J Virol* **77**, 4502-4515.

Cheung, A. K., Gottlieb, D. J., Plachter, B., Pepperl-Klindworth, S., Avdic, S., Cunningham, A. L., Abendroth, A. & Slobedman, B. (2009). The role of the human cytomegalovirus UL111A gene in down-regulating CD4+ T-cell recognition of latently infected cells: implications for virus elimination during latency. *Blood* **114**, 4128-4137.

Couper, K. N., Blount, D. G. & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771-5777.

D'Andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M. & Trinchieri, G. (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* **178**, 1041-1048.

Davison, A., Holton, M., Dolan, A., Dargan, D., Gatherer, D. & Hayward, G. (2013). Comparative genomics of primate cytomegaloviruses. In *Cytomegaloviruses: from molecular pathogenesis to intervention*. Edited by Matthias J Reddehase.

de Lemos Rieper, C., Galle, P., Pedersen, B. K. & Hansen, M. B. (2011). Characterization of specific antibodies against cytomegalovirus (CMV)-encoded interleukin 10 produced by 28% of CMV-seropositive blood donors. *J Gen Virol* **92**, 1508-1518.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. (1991a). Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174**, 1209-1220.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. & other authors (1991b). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* **174**, 915-924.

Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F. & Banchereau, J. (1992). Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* **175**, 671-682.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M. G., Biagiotti, R. & Romagnani, S. (1993). Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* **150**, 353-360.

Delhon, G., Tulman, E., Afonso, C., Lu, Z., De la Concha-Bermejillo, A., Lehmkuhl, H., Piccone, M., Kutish, G. & Rock, D. (2004). Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. *J Virol* **78**, 168-177.

Demangel, C., Bertolino, P. & Britton, W. J. (2002). Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur J Immunol* **32**, 994-1002.

Díaz-San Segundo, F., Rodríguez-Calvo, T., de Avila, A. & Sevilla, N. (2009). Immunosuppression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. *PLoS One* **4**, e5659.

Ding, Y., Qin, L., Kotenko, S. V., Pestka, S. & Bromberg, J. S. (2000). A single amino acid determines the immunostimulatory activity of interleukin 10. *J Exp Med* **191**, 213-224.

Ding, Y., Qin, L., Zamarin, D., Kotenko, S. V., Pestka, S., Moore, K. W. & Bromberg, J. S. (2001). Differential IL-10R1 expression plays a critical role in IL-10-mediated immune regulation. *J Immunol* **167**, 6884-6892.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Zhu, H. & Liu, F. (2003). Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* **100**, 14223-14228.

Eberhardt, M. K., Chang, W. L., Logsdon, N. J., Yue, Y., Walter, M. R. & Barry, P. A. (2012). Host immune responses to a viral immune modulating protein: immunogenicity of viral interleukin-10 in rhesus cytomegalovirus-infected rhesus macaques. *PLoS One* **7**, e37931.

Eberhardt, M. K., Deshpande, A., Chang, W. W., Barthold, S. W., Walter, M. R. & Barry, P. A. (2013). Vaccination against a virus-encoded cytokine significantly restricts viral challenge. *J Virol* **87**, 11323-11331.

Fiorentino, D. F., Bond, M. W. & Mosmann, T. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* **170**, 2081-2095.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147**, 3815-3822.

Fleming, S. B., McCaughan, C. A., Andrews, A. E., Nash, A. D. & Mercer, A. A. (1997). A homolog of interleukin-10 is encoded by the poxvirus orf virus. *J Virol* **71**, 4857-4861.

Fleming, S. B., Haig, D. M., Nettleton, P., Reid, H. W., McCaughan, C. A., Wise, L. M. & Mercer, A. (2000). Sequence and functional analysis of a homolog of interleukin-10 encoded by the parapoxvirus orf virus. *Virus genes* **21**, 85-95.

Fleming, S. B., Anderson, I. E., Thomson, J., Deane, D. L., McInnes, C. J., McCaughan, C. A., Mercer, A. A. & Haig, D. M. (2007). Infection with recombinant orf viruses demonstrates that the viral interleukin-10 is a virulence factor. *J Gen Virol* **88**, 1922-1927.

Franken, M., Devergne, O., Rosenzweig, M., Annis, B., Kieff, E. & Wang, F. (1996). Comparative analysis identifies conserved tumor necrosis factor receptor-associated factor 3 binding sites in the human and simian Epstein-Barr virus oncogene LMP1. *J Virol* **70**, 7819-7826.

Gad, H. H., Dellgren, C., Hamming, O. J., Vends, S., Paludan, S. R. & Hartmann, R. (2009). Interferon-λ is functionally an interferon but structurally related to the interleukin-10 family. *J Biol Chem* **284**, 20869-20875.

Go, N. F., Castle, B. E., Barrett, R., Kastelein, R., Dang, W., Mosmann, T. R., Moore, K. W. & Howard, M. (1990). Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med* **172**, 1625-1631.

Haig, D. M., Thomson, J., McInnes, C., McCaughan, C., Imlach, W., Mercer, A. & Fleming, S. (2002a). Orf virus immuno-modulation and the host immune response. *Vet Immunol Immunopathol* **87**, 395-399.

Haig, D. M., Thomson, J., McInnes, C. J., Deane, D. L., Anderson, I. E., McCaughan, C. A., Imlach, W., Mercer, A. A., Howard, C. J. & other authors (2002b). A comparison of the anti-inflammatory and immuno-stimulatory activities of orf virus and ovine interleukin-10. *Virus Res* **90**, 303-316.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houlès, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J. F. & other authors (2002). Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity* **17**, 653-664.

Hart, P. H., Hunt, E. K., Bonder, C. S., Watson, C. J. & Finlay-Jones, J. J. (1996). Regulation of surface and soluble TNF receptor expression on human monocytes and synovial fluid macrophages by IL-4 and IL-10. *J Immunol* **157**, 3672-3680.

Hautaniemi, M., Ueda, N., Tuimala, J., Mercer, A. A., Lahdenperä, J. & McInnes, C. J. (2010). The genome of pseudocowpoxvirus: comparison of a reindeer isolate and a reference strain. *J Gen Virol* **91**, 1560-1576.

Hsu, D. H., de Waal Malefyt, R., Fiorentino, D. F., Dang, M. N., Vieira, P., de Vries, J., Spits, H., Mosmann, T. R. & Moore, K. W. (1990). Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *Science* **250**, 830-832.

Hudson, G. S., Bankier, A. T., Satchwell, S. C. & Barrell, B. G. (1985). The short unique region of the B95-8 Epstein-Barr virus genome. *Virology* **147**, 81-98.

Hwu, P., Du, M. X., Lapointe, R., Taylor, M. W. & Young, H. A. (2000). Indoleamine 2, 3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol* **164**, 3596-3599.

Ilouze, M., Dishon, A. & Kotler, M. (2012). Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. *Virus Res* **169**, 98-106.

Imlach, W., McCaughan, C. A., Mercer, A. A., Haig, D. & Fleming, S. B. (2002). Orf virus-encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits cytokine synthesis in murine peritoneal macrophages. *J Gen Virol* **83**, 1049-1058.

Isfort, R., Jones, D., Kost, R., Witter, R. & Kung, H. J. (1992). Retrovirus insertion into herpesvirus in vitro and in vivo. *Proc Natl Acad Sci U S A* **89**, 991-995.

Jaworowski, A., Cheng, W. J., Westhorpe, C. L., Abendroth, A., Crowe, S. M. & Slobedman, B. (2009). Enhanced monocyte Fc phagocytosis by a homologue of interleukin-10 encoded by human cytomegalovirus. *Virology* **391**, 20-24.

Jayawardane, G., Russell, G. C., Thomson, J., Deane, D., Cox, H., Gatherer, D., Ackermann, M., Haig, D. M. & Stewart, J. P. (2008). A captured viral interleukin 10 gene with cellular exon structure. *J Gen Virol* **89**, 2447-2455.

Jenkins, J., Malyak, M. & Arend, W. (1994). The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. *Lymphokine Cytokine Res* **13**, 47.

Jenkins, C., Abendroth, A. & Slobedman, B. (2004). A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J Virol* **78**, 1440-1447.

Jenkins, C., Garcia, W., Abendroth, A. & Slobedman, B. (2008a). Expression of a human cytomegalovirus latency-associated homolog of interleukin-10 during the productive phase of infection. *Virology* **370**, 285-294.

Jenkins, C., Garcia, W., Godwin, M. J., Spencer, J. V., Stern, J. L., Abendroth, A. & Slobedman, B. (2008b). Immunomodulatory properties of a viral homolog of human interleukin-10 expressed by human cytomegalovirus during the latent phase of infection. *J Virol* **82**, 3736-3750.

Jochum, S., Moosmann, A., Lang, S., Hammerschmidt, W. & Zeidler, R. (2012). The EBV immunoevasins vIL-10 and BNLF2a Protect newly infected B cells from immune recognition and elimination. *PLoS Pathog* **8**, e1002704.

Jones, B. C., Logsdon, N. J., Josephson, K., Cook, J., Barry, P. A. & Walter, M. R. (2002). Crystal structure of human cytomegalovirus IL-10 bound to soluble human IL-10R1. *Proc Natl Acad Sci U S A* **99**, 9404-9409.

Josephson, K., Logsdon, N. J. & Walter, M. R. (2001). Crystal structure of the IL-10/IL-10R1 complex reveals a shared receptor binding site. *Immunity* **15**, 35-46.

Keravala, A., Lechman, E. R., Nash, J., Mi, Z. & Robbins, P. D. (2006). Human, viral or mutant human IL-10 expressed after local adenovirus-mediated gene transfer are equally effective in ameliorating disease pathology in a rabbit knee model of antigen-induced arthritis. *Arthritis Res Ther* **8**, R91.

Kim, K. N., Watanabe, S., Ma, Y., Thornton, S., Giannini, E. H. & Hirsch, R. (2000). Viral IL-10 and soluble TNF receptor act synergistically to inhibit collagen-induced arthritis following adenovirus-mediated gene transfer. *J Immunol* **164**, 1576-1581.

Kotenko, S. V. & Pestka, S. (2001). Viral IL-10 variants. Cytokine Reference, Two-Volume Set (Individual Version): A Compendium of Cytokines and Other Mediators of Host Defense. In Cytokine Reference, pp.1-13. Edited by F. Marc., D. Scott K., H. Toshio., V. Jan ., N. Nicos A.,O. Joost J. Academic Press.

Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V. & Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci U S A* **97**, 1695-1700.

Kotenko, S. V., Krause, C. D., Izotova, L. S., Pollack, B. P., Wu, W. & Pestka, S. (1997). Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J* **16**, 5894-5903.

Lateef, Z., Fleming, S., Halliday, G., Faulkner, L., Mercer, A. & Baird, M. (2003). Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. *J Gen Virol* **84**, 1101-1109.

Lechman, E. R., Jaffurs, D., Ghivizzani, S. C., Gambotto, A., Kovesdi, I., Mi, Z., Evans, C. H. & Robbins, P. D. (1999). Direct adenoviral gene transfer of viral IL-10 to rabbit knees with experimental arthritis ameliorates disease in both injected and contralateral control knees. *J Immunol* **163**, 2202-2208.

Lee, H. J., Essani, K. & Smith, G. L. (2001). The genome sequence of Yaba-like disease virus, a yatapoxvirus. *Virology* **281**, 170-192.

Lin, Y. L., Chang, P. C., Wang, Y. & Li, M. (2008). Identification of novel viral interleukin-10 isoforms of human cytomegalovirus AD169. *Virus Res* **131**, 213-223.

Liu, Y., Wei, S. H., Ho, A. S., de Waal Malefyt, R. & Moore, K. W. (1994). Expression cloning and characterization of a human IL-10 receptor. *J Immunol* **152**, 1821-1829.

Liu, Y., de Waal Malefyt, R., Briere, F., Parham, C., Bridon, J. M., Banchereau, J., Moore, K. W. & Xu, J. (1997). The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor. *J Immunol* **158**, 604-613.

Lockridge, K. M., Zhou, S. S., Kravitz, R. H., Johnson, J. L., Sawai, E. T., Blewett, E. L. & Barry, P. A. (2000). Primate cytomegaloviruses encode and express an IL-10-like protein. *Virology* **268**, 272-280.

Logsdon, N. J., Eberhardt, M. K., Allen, C. E., Barry, P. A. & Walter, M. R. (2011). Design and analysis of rhesus cytomegalovirus IL-10 mutants as a model for novel vaccines against human cytomegalovirus. *PloS One* **6**, e28127.

Ma, Y., Thornton, S., Duwel, L. E., Boivin, G. P., Giannini, E. H., Leiden, J. M., Bluestone, J. A. & Hirsch, R. (1998). Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. *J Immunol* **161**, 1516-1524.

Matsuda, M., Salazar, F., Petersson, M., Masucci, G., Hansson, J., Pisa, P., Zhang, Q. J., Masucci, M. G. & Kiessling, R. (1994). Interleukin 10 pretreatment protects target cells from tumor- and allo-specific cytotoxic T cells and downregulates HLA class I expression. *J Exp Med* **180**, 2371-2376.

Meier-Trummer, C. S., Tobler, K., Hilbe, M., Stewart, J. P., Hart, J., Campbell, I., Haig, D. M., Glauser, D. L., Ehrensperger, F. & other authors (2009). Ovine herpesvirus 2 structural proteins in epithelial cells and M-cells of the appendix in rabbits with malignant catarrhal fever. *Vet Microbiol* **137**, 235-242.

Miyazaki, I., Cheung, R. K. & Dosch, H. M. (1993). Viral interleukin 10 is critical for the induction of B cell growth transformation by Epstein-Barr virus. *J Exp Med* **178**, 439-447.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683-765.

Moore, K. W., O'Garra, A., Malefyt, R. W., Vieira, P. & Mosmann, T. R. (1993). Interleukin-10. *Annu Rev Immunol* **11**, 165-190.

Moore, K. W., Vieira, P., Fiorentino, D. F., Trounstine, M. L., Khan, T. A. & Mosmann, T. R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science* **248**, 1230-1234.

Mosser, D. M. & Zhang, X. (2008). Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* **226**, 205-218.

Nachtwey, J. & Spencer, J. V. (2008). HCMV IL-10 suppresses cytokine expression in monocytes through inhibition of nuclear factor-κ B. *Viral Immunol* **21**, 477-482.

Nast, C. C., Moudgil, A., Zuo, X. J., Toyoda, M. & Jordan, S. C. (1997). Long-term allograft acceptance in a patient with posttransplant lymphoproliferative disorder: correlation with intragraft viral interleukin-10. *Transplantation* **64**, 1578-1582.

Niiro, H., Otsuka, T., Abe, M., Satoh, H., Ogo, T., Nakano, T., Furukawa, Y. & Niho, Y. (1992). Epstein-Barr virus BCRF1 gene product (viral interleukin 10) inhibits superoxide anion production by human monocytes. *Lymphokine Cytokine Res* **11**, 209-214.

Niiro, H., Otsuka, T., Kuga, S., Nemoto, Y., Abe, M., Hara, N., Nakano, T., Ogo, T. & Niho, Y. (1994). IL-10 inhibits prostaglandin E2 production by lipopolysaccharide-stimulated monocytes. *Int Immunol* **6**, 661-664.

Odom, M. R., Hendrickson, R. C. & Lefkowitz, E. J. (2009). Poxvirus protein evolution: family wide assessment of possible horizontal gene transfer events. *Virus Res* **144**, 233-249.

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A. & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **29**, 71-109.

Ouyang, P., Rakus, K., Boutier, M., Reschner, A., Leroy, B., Ronsmans, M., Fournier, G., Scohy, S., Costes, B. & other authors (2013). The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. *Vet Res* **44**, 53.

Pepperl-Klindworth, S., Besold, K., Frankenberg, N., Farkas, M., Kuball, J., Theobald, M. & Plachter, B. (2006). Cytomegalovirus interleukin-10 expression in infected cells does not impair MHC class I restricted peptide presentation on bystanding antigen-presenting cells. *Viral Immunol* **19**, 92-101.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. & Fisher, P. B. (2004). Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* **22**, 929-979.

Pinto, R. D., Nascimento, D. S., Reis, M. I., do Vale, A. & Dos Santos, N. M. (2007). Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10. *Mol Immunol* **44**, 2056-2065.

Qin, L., Chavin, K. D., Ding, Y., Tahara, H., Favaro, J. P., Woodward, J. E., Suzuki, T., Robbins, P. D., Lotze, M. T. & other authors (1996). Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J Immunol* **156**, 2316-2323.

Raftery, M. J., Wieland, D., Gronewald, S., Kraus, A. A., Giese, T. & Schönrich, G. (2004). Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. *J Immunol* **173**, 3383-3391.

Raftery, M. J., Hitzler, M., Winau, F., Giese, T., Plachter, B., Kaufmann, S. H. E. & Schönrich, G. (2008). Inhibition of CD1 antigen presentation by human cytomegalovirus. *J Virol* **82**, 4308-4319.

Rouas-Freiss, N., Gonçalves, R. M. B., Menier, C., Dausset, J. & Carosella, E. D. (1997). Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci U S A* **94**, 11520-11525.

Rousset, F., Garcia, E., Defrance, T., Péronne, C., Vezzio, N., Hsu, D. H., Kastelein, R., Moore, K. W. & Banchereau, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* **89**, 1890-1893.

Rowbottom, A., Lepper, M., Garland, R., Cox, C., Corley, E., Oakhill, A. & Steward, C. (1999). Interleukin-10-induced CD8 cell proliferation. *Immunology* **98**, 80-89.

Sabat, R. (2010). IL-10 family of cytokines. *Cytokine Growth Factor Rev* **21**, 315-324.

Sabat, R., Grutz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K. & Geginat, J. (2010). Biology of interleukin-10. *Cytokine Growth Factor Rev* **21**, 331-344.

Salek-Ardakani, S., Arrand, J. R. & Mackett, M. (2002a). Epstein-Barr virus encoded interleukin-10 inhibits HLA-class I, ICAM-1, and B7 expression on human monocytes: implications for immune evasion by EBV. *Virology* **304**, 342-351.

Salek-Ardakani, S., Stuart, A. D., Arrand, J. E., Lyons, S., Arrand, J. R. & Mackett, M. (2002b). High level expression and purification of the Epstein-Barr virus encoded cytokine viral interleukin 10: efficient removal of endotoxin. *Cytokine* **17**, 1-13.

Santin, A. D., Hermonat, P. L., Ravaggi, A., Bellone, S., Pecorelli, S., Roman, J. J., Parham, G. P. & Cannon, M. J. (2000). Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8+ cytotoxic T lymphocytes. *J Virol* **74**, 4729-4737.

Shackelton, L. A. & Holmes, E. C. (2004). The evolution of large DNA viruses: combining genomic information of viruses and their hosts. *Trends Microbiol* **12**, 458-465.

Slobedman, B., Barry, P. A., Spencer, J. V., Avdic, S. & Abendroth, A. (2009). Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. *J Virol* **83**, 9618-9629.

Spencer, J. V. (2007). The cytomegalovirus homolog of interleukin-10 requires phosphatidylinositol 3-kinase activity for inhibition of cytokine synthesis in monocytes. *J Virol* **81**, 2083-2086.

Spencer, J. V., Cadaoas, J., Castillo, P. R., Saini, V. & Slobedman, B. (2008). Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10. *Virology* **374**, 164-169.

Spencer, J. V., Lockridge, K. M., Barry, P. A., Lin, G., Tsang, M., Penfold, M. E. T. & Schall, T. J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J Virol* **76**, 1285-1292.

Stuart, A. D., Stewart, J. P., Arrand, J. R. & Mackett, M. (1995). The Epstein-Barr virus encoded cytokine viral interleukin-10 enhances transformation of human B lymphocytes. *Oncogene* **11**, 1711-1720.

Sunarto, A., Liongue, C., McColl, K. A., Adams, M. M., Bulach, D., Crane, M. S. J., Schat, K. A., Slobedman, B., Barnes, A. C. & other authors (2012). Koi herpesvirus encodes and expresses a functional interleukin-10. *J Virol* **86**, 11512-11520.

Takanaski, S., Nonaka, R., Xing, Z., O'Byrne, P., Dolovich, J. & Jordana, M. (1994). Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med* **180**, 711-715.

Telford, E. A. R., Watson, M. S., Aird, H. C., Perry, J. & Davison, A. J. (1995). The DNA sequence of equine herpesvirus 2. *J Mol Biol* **249**, 520-528.

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W. & Rennick, D. M. (1991). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *J Exp Med* **173**, 507-510.

Touitou, R., Cochet, C. & Joab, I. (1996). Transcriptional analysis of the Epstein-Barr virus interleukin-10 homologue during the lytic cycle. *J Gen Virol* **77 (Pt 6)**, 1163-1168.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Kutish, G. & Rock, D. (2001). Genome of lumpy skin disease virus. *J Virol* **75**, 7122-7130.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Kutish, G. & Rock, D. (2004). The genome of canarypox virus. *J Virol* **78**, 353-366.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Sur, J. H., Sandybaev, N., Kerembekova, U., Zaitsev, V., Kutish, G. & other authors (2002). The genomes of sheeppox and goatpox viruses. *J Virol* **76**, 6054-6061.

van Beurden, S. J., Peeters, B. P., Rottier, P. J., Davison, A. J. & Engelsma, M. Y. (2013). Genome-wide gene expression analysis of anguillid herpesvirus 1. *BMC Genomics* **14**, 83.

van Beurden, S. J., Forlenza, M., Westphal, A. H., Wiegertjes, G. F., Haenen, O. L. & Engelsma, M. Y. (2011). The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish Shellfish Immunol* **31**, 1211-1217.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

van Putten, S., Wübben, M., Hennink, W., van Luyn, M. & Harmsen, M. (2009). The downmodulation of the foreign body reaction by cytomegalovirus encoded interleukin-10. *Biomaterials* **30**, 730-735.

Vieira, P., de Waal-Malefyt, R., Dang, M., Johnson, K., Kastelein, R., Fiorentino, D., Roncarolo, M., Mosmann, T. & Moore, K. (1991). Isolation and expression of human cytokine synthesis

inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc Natl Acad Sci U S A* **88**, 1172-1176.

Walter, M. R. & Nagabhushan, T. L. (1995). Crystal structure of interleukin 10 reveals an interferon gamma-like fold. *Biochemistry* **34**, 12118-12125.

Whalen, J. D., Lechman, E. L., Carlos, C. A., Weiss, K., Glorioso, J. C., Robbins, P. D. & Evans, C. H. (1999). Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J Immunol* **162**, 3625-3632.

Willems, F., Marchant, A., Delville, J. P., Gérard, C., Delvaux, A., Velu, T., De Boer, M. & Goldman, M. (1994). Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur J Immunol* **24**, 1007-1009.

Wise, L., McCaughan, C., Tan, C. K., Mercer, A. A. & Fleming, S. B. (2007). Orf virus interleukin-10 inhibits cytokine synthesis in activated human THP-1 monocytes, but only partially impairs their proliferation. *J Gen Virol* **88**, 1677-1682.

Wolk, K., Witte, E., Reineke, U., Witte, K., Friedrich, M., Sterry, W., Asadullah, K., Volk, H. & Sabat, R. (2004). Is there an interaction between interleukin-10 and interleukin-22? *Genes Immun* **6**, 8-18.

Xu, Z., Iwatsuki, K., Oyama, N., Ohtsuka, M., Satoh, M., Kikuchi, S., Akiba, H. & Kaneko, F. (2001). The latency pattern of Epstein–Barr virus infection and viral IL-10 expression in cutaneous natural killer/T-cell lymphomas. *Br J Cancer* **84**, 920.

Yamamoto-Tabata, T., McDonagh, S., Chang, H. T., Fisher, S. & Pereira, L. (2004). Human cytomegalovirus interleukin-10 downregulates metalloproteinase activity and impairs endothelial cell migration and placental cytotrophoblast invasiveness in vitro. *J Virol* **78**, 2831-2840.

Yoon, S. I., Jones, B. C., Logsdon, N. J. & Walter, M. R. (2005). Same Structure, Different Function: Crystal Structure of the Epstein-Barr Virus IL-10Bound to the Soluble IL-10R1 Chain. *Structure* **13**, 551-564.

Yoon, S. I., Logsdon, N. J., Sheikh, F., Donnelly, R. P. & Walter, M. R. (2006). Conformational changes mediate interleukin-10 receptor 2 (IL-10R2) binding to IL-10 and assembly of the signaling complex. *J Biol Chem* **281**, 35088-35096.

Yu, X., Cheng, Y., Shi, B., Qian, F., Wang, F., Liu, X., Yang, H., Xu, Q., Qi, T. & other authors (2008). Measles virus infection in adults induces production of IL-10 and is associated with increased $CD4^+$ $CD25^+$ regulatory T cells. *J Immunol* **181**, 7356-7366.

Yue, Y. & Barry, P. A. (2008). Rhesus cytomegalovirus: a nonhuman primate model for the study of human cytomegalovirus. *Adv Virus Res* **72**, 207-226.

Zdanov, A. (2004). Structural features of the interleukin-10 family of cytokines. *Curr Pharm Des* **10**, 3873-3884.

Zdanov, A., Schalk-Hihi, C. & Wlodawer, A. (1996). Crystal structure of human interleukin-10 at 1.6 Å resolution and a model of a complex with its soluble receptor. *Protein Sci* **5**, 1955-1962.

Zdanov, A., Schalk-Hihi, C., Menon, S., Moore, K. W. & Wlodawer, A. (1997). Crystal structure of Epstein-Barr virus protein BCRF1, a homolog of cellular interleukin-10. *J Mol Biol* **268**, 460-467.

Zdanov, A., Schalk-Hihi, C., Gustchina, A., Tsang, M., Weatherbee, J. & Wlodawer, A. (1995). Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. *Structure* **3**, 591-601.

Zhang, D. C., Shao, Y. Q., Huang, Y. Q. & Jiang, S. G. (2005). Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerion*). *J Biochem Mol Biol* **38**, 571-576.

Table S1. Sequences used to build trees[1]

Protein	Host species		Accession number[2]	Fig. 3	Fig. 4
IL-10	Xenopus	*Xenopus (Silurana) tropicalis*	ref|NP_001165400.1	X	X
	Rock dove	*Columba livia*	gb|EMC81973.1	X	X
	Chicken	*Gallus gallus*	ref|NP_001004414.2	X	X
	Green seaturtle	*Chelonia mydas*	gb|EMP30816.1	X	X
	Tasmanian devil	*Sarcophilus harrisii*	ref|XP_003767694.1	X	X
	Opossum	*Monodelphis domestica*	ref|XP_003340215.1	X	X
	Cat	*Felis catus*	ref|NP_001009209.1	X	X
	Walrus	*Odobenus rosmarus divergens*	ref|XP_004417638.1	X	X
	Red deer	*Cervus elaphus*	sp|P51746.1|IL10_CEREL	X	X
	Sheep	*Ovis aries*	emb|CAG38358.1	X	X
	Goat	*Capra hircus*	gb|ABI20513.1	X	X
	Cattle	*Bos taurus*	ref|NP_776513.1	X	X
	Chinese hamster	*Cricetulus griseus*	ref|XP_003501267.1	X	X
	Mouse	*Mus musculus*	ref|NP_034678.1	X	X
	Rabbit	*Oryctolagus cuniculus*	gb|ABC41664.1	X	X
	Marmoset	*Callithrix jacchus*	ref|XP_002760779.1	X	X
	Human	*Homo sapiens*	ref|NP_000563.1	X	X
	Chimpanzee	*Pan troglodytes*	ref|NP_001129092.2	X	X
	Bonobo	*Pan paniscus*	ref|XP_003822966.1	X	X
	Gorilla	*Gorilla gorilla gorilla*	ref|XP_004028338.1	X	X
	Baboon	*Papio anubis*	ref|XP_003893246.1	X	X
	Macaque	*Macaca mulatta*	ref|NP_001038192.1	X	X
	Bottlenose dolphin	*Tursiops truncatus*	ref|XP_004312277.1	X	X
	Pig	*Sus scrofa*	gb|ABP68816.1	X	X
	Horse	*Equus caballus*	ref|NP_001075959.1	X	X
	Myotis	*Myotis davidii*	gb|ELK37201.1	X	X
	African elephant	*Loxodonta africana*	ref|XP_003410325.1	X	X
	Chinese treeshrew	*Tupaia chinensis*	gb|ELW47753.1	X	X
	Galago	*Otolemur garnettii*	ref|XP_003792257.1	X	X
	Zebrafish	*Danio rerio*	ref|NP_001018621.2	X	X
	Common carp	*Cyprinus carpio*	dbj|BAC76885.1	X	X
	European eel	*Anguilla anguilla*	gb|AEL99923.1	X	X
IL-19	Human	*Homo sapiens*	IL19_HUMAN	X	
	Mouse	*Mus musculus*	IL19_MOUSE	X	
IL-20	Human	*Homo sapiens*	IL20_HUMAN	X	
	Mouse	*Mus musculus*	IL20_MOUSE	X	
IL-22	Human	*Homo sapiens*	IL22_HUMAN	X	
	Mouse	*Mus musculus*	IL22_MOUSE	X	
IL-22B	Mouse	*Mus musculus*	IL22B_MOUSE	X	
IL-24	Human	*Homo sapiens*	IL24_HUMAN	X	
	Mouse	*Mus musculus*	IL24_MOUSE	X	
	Rat	*Rattus norvegicus*	IL24_RAT	X	
IL-26	Human	*Homo sapiens*	IL26_HUMAN	X	
Protein	**Virus**		**Accession number**	**Fig. 3**	**Fig. 4**
134R	Yaba-like disease virus		Q9DHH9	X	

[1] Sequences listed in Table 1 were also used to build the trees presented in Figure 3 and Figure 4.

[2] Accession numbers for IL-19, IL-20, IL-22, IL-22B, IL-24, IL-26 and 134R are from SwissProt. Other accession numbers are from GenBank.

Supplementary material S1

Methods and sequences used to build the trees presented in Figure 3 and Figure 4.

Methods

The membership of the IL-10 protein family was defined according to Pfam (Punta *et al.*, 2012) (http://pfam.sanger.ac.uk/family/IL10, PF00726). As well as the "true" IL-10 proteins, this protein family includes the related IL-19, IL-20, IL-22, IL-24 and IL-26 proteins. Protein sequences from the IL-10 family were loaded into MEGA (Tamura *et al.*, 2011) and aligned using Muscle (Edgar, 2004). The best-fitting substitution model was derived and found to be the Jones-Taylor-Thornton model with a gamma distribution of rates among sites (JTT+Γ). A maximum likelihood phylogenetic tree was then built using that model. Bayesian phylogenetic trees were built using BEAST (Drummond & Rambaut, 2007), run on the same substitution models until convergence. The tree prior was a speciating Yule model, and a relaxed exponential clock was found to be the best-fitting clock model, by comparison of different models using Bayes factors within BEAST.

References

Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7**, 214.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-1797.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A. & Finn, R. D. (2012). The Pfam protein families database. *Nucleic Acids Res* **40**, D290-301.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-2739.

Supplementary material S2

Rational for considering the IL-10 orthologue found in the bonobo XP_003804206.1 sequence as a sequence deriving from a contaminating bonobo herpesvirus

The *Pan paniscus* vIL-10 sequence was found in the locus XP_003804206 http://www.ncbi.nlm.nih.gov/protein/397464698. A nucleotide alignment of the *Pan paniscus* unplaced genomic scaffold panpan1 scf1120388612767 (an almost 5kb DNA fragment, which harbours this vIL-10 gene) with the closely related Epstein-Barr virus suggesting that the whole scaffold is indeed viral. However, the viral sequence was too distant from EBV to represent a strain of this viral species. Most likely, this virus represents a bonobo lymphocryptovirus, probably the one for which a partial sequence of the DNA polymerase and glycoprotein B were published by Ehlers at al., 2010. Indeed, searching of the DNA pol and gB sequence in the *Pan paniscus* ENA files, lead to the retrieve of another 4766 bp sequence (AJFE01005652.1: Pan paniscus cntg5853, http://www.ebi.ac.uk/ena/data/view/AJFE01005652&range=2-1842), which is 97% similar over >3kb to the pan paniscus lymphocryptovirus 1 described by Ehlers et al. (2010) (and ~88% to EBV). It is very likely that most of the sequences of this bonobo herpesvirus (with the exception of the vIL-10) were removed from the bonobo genome assembly, because they were mistakenly seen as a contamination by EBV which was used in the cell-line transformation.

References

Ehlers, B., Spiess, K., Leendertz, F., Peeters, M., Boesch, C., Gatherer, D. & McGeoch, D. J. (2010). Lymphocryptovirus phylogeny and the origins of Epstein-Barr virus. *J Gen Virol* **91**, 630-642.

Objectives

Objectives

Cyprinid herpesvirus 3 is the etiological agent of an emerging and mortal disease in common (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) carp. Since its emergence, in the late 1990s, this highly contagious and dreadful disease has caused severe economic losses in both common and koi carp culture industries worldwide.

In addition to its economic importance, CyHV-3 has several qualities as a fundamental model of infection: (i) It is phylogenically distant from the vast majority of herpesviruses that have been studied so far. (ii) It can be studied in laboratories by infection of its natural host (homologous virus-host model). (iii) The sequence of its genome published in 2007 revealed a fascinating virus with unique properties in the *Herpesvirales*, such as an extremely large genome (295 Kb), a high number of genes which are not homologous to known viral sequences, and genes that are normally found exclusively in the *Poxviridae*. (iv) Interestingly, the sequencing of the CyHV-3 genome revealed several genes potentially encoding proteins involved in immune evasion mechanisms. Among these genes, ORF134 encodes an orthologue of carp Interleukin 10 (IL-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 properties. Many viruses exploit the immunosuppressive properties of IL-10 to evade immune recognition either by up-regulation of cellular IL-10 or by expressing viral orthologues of cellular IL-10 called viral IL-10 (vIL-10). vIL-10s have been identified in several members of the *Poxviridae* family and the *Herpesvirales* order. The biological activities of various vIL-10s have been studied *in vitro* by using recombinant proteins and have demonstrated their broad immunosuppressive spectrum. Moreover, for few viruses (RhCMV and ORFV) encoding vIL-10s, it has been shown that deletion of the coding gene was associated with virus attenuation *in vivo*.

In the present thesis, we took profit of the identification of a vIL-10 gene in the genome of CyHV-3 to study the roles of such a protein in the biology of the infection of an *Alloherpesviridae* family member both *in vitro* and *in vivo*.

Experimental section

Experimental section

1st chapter:

The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication *in vitro* nor for virulence *in vivo*

Veterinary Research (2013), 44:53

Ping Ouyang[1], Krzysztof Rakus[1], Maxime Boutier[1], Anca Reschner[1], Baptiste Leroy[2], Maygane Ronsmans[1], Guillaume Fournier[1], Sophie Scohy[3], Bérénice Costes[4], Ruddy Wattiez[2] and Alain Vanderplasschen[1*]

[1]Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases (B43b), Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium
[2]Proteomic and Microbiology, CISMa, University of Mons, Place du parc 20 B-7000 Mons, Belgium
[3]Delphi Genetics SA, rue Antoine de Saint-Exupéry, 5, B-6041 Gosselies, Belgium
[4]Femalon S.A., Rue du Travail, 16, B-4460 Grâce-Hollogne, Belgium

* Corresponding author. E-mail: a.vdplasschen@ulg.ac.be. Phone: +32 4 366 42 64

Preamble

For millions of years, viruses have been co-evolving with their hosts. During this co-evolution process, viruses have evolved elegant mechanisms to evade detection and destruction by the host immune system. One of the evasion strategies that have been adopted by large DNA viruses is to encode homologues of cytokines, chemokines and their receptors — molecules that have a crucial role in control of the immune response.

Cellular Interleukine-10 (cIL-10) is a pleiotropic cytokine, with both immunostimulating and immunosuppressive properties; however, the key features of this cytokine relate mainly to its capacity to exert potent immunosuppressive functions through various mechanisms. Many viruses exploit the immunosuppressive properties of IL-10 to evade immune recognition either by up-regulation of host IL-10 or by expression of virally encoded IL-10 homologues.

Virally encoded IL-10 homologues have been reported in several members of the *Poxviridae* family and the *Herpesvirales* order. Among the *Herpesvirales* order, vIL-10s have been described in members of the *Herpesviridae* (e.g. human cytomegalovirus [HCMV] and Epstein-Barr virus [EBV]) and more recently in the family *Alloherpesviridae* (Anguilid herpesvirus 1 [AngHV-1] and CyHV-3). While the role of vIL-10s has been demonstrated in the pathogenesis of only one member of family *Poxviridae* and *Herpesviridae*, respectively; this has not yet been investigated in the member of family *Alloherpesviridae*.

In the present study, we investigated the roles of CyHV-3 ORF134 in the biology of the infection *in vitro* and *in vivo. In vitro* studies demonstrated that ORF134 is expressed as a spliced early-late gene and that its expression product is the second most abundant viral protein in the CyHV-3 secretome. Taking advantage of the recent BAC cloning of CyHV-3 as an infectious bacterial artificial chromosome (BAC), a strain deleted for ORF134 and a derived revertant strain were produced. Comparison of these strains demonstrated that ORF134 is essential neither for CyHV-3 replication *in vitro* nor for virulence *in vivo*.

Ouyang et al. Veterinary Research 2013, 44:53
http://www.veterinaryresearch.org/content/44/1/53

VETERINARY RESEARCH

RESEARCH Open Access

The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo

Ping Ouyang[1], Krzysztof Rakus[1], Maxime Boutier[1], Anca Reschner[1], Baptiste Leroy[2], Maygane Ronsmans[1], Guillaume Fournier[1], Sophie Scohy[3], Bérénice Costes[4], Ruddy Wattiez[2] and Alain Vanderplasschen[1*]

Abstract

Cyprinid herpesvirus 3 (CyHV-3), a member of the family *Alloherpesviridae*, is the causative agent of a lethal disease in common and koi carp. CyHV-3 ORF134 encodes an interleukin-10 (IL-10) homologue. The present study was devoted to this ORF. Transcriptomic analyses revealed that ORF134 is expressed as a spliced gene belonging to the early-late class. Proteomic analyses of CyHV-3 infected cell supernatant demonstrated that the ORF134 expression product is one of the most abundant proteins of the CyHV-3 secretome. To investigate the role of ORF134 in viral replication in vitro and in virulence in vivo, a deleted strain and a derived revertant strain were produced using BAC cloning technologies. The recombinant ORF134 deleted strain replicated in vitro comparably to the parental and the revertant strains. Infection of fish by immersion in water containing the virus induced comparable CyHV-3 disease for the three virus genotypes tested (wild type, deleted and revertant). Quantification of viral DNA by real time TaqMan PCR (in the gills and the kidney) and analysis of carp cytokine expression (in the spleen) by RT-qPCR at different times post-infection did not revealed any significant difference between the groups of fish infected with the three virus genotypes. Similarly, histological examination of the gills and the kidney of infected fish revealed no significant differences between fish infected with ORF134 deleted virus versus fish infected with the control parental or revertant strains. All together, the results of the present study demonstrate that the IL-10 homologue encoded by CyHV-3 is essential neither for viral replication in vitro nor for virulence in common carp.

Introduction

Koi herpesvirus (KHV), also known as cyprinid herpesvirus 3 (CyHV-3; species *Cyprinid herpesvirus 3*, genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), is the etiological agent of an emerging and mortal disease in common (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) carp [1,2]. Since its emergence, in the late 1990s, this highly contagious and dreadful disease has caused severe economic losses in both common and koi carp culture industries worldwide [3-5].

The genome of CyHV-3 comprises a linear double-stranded DNA sequence of ~295 kbp [6], similar to that of cyprinid herpesvirus 1 and 2 (CyHV-1 and CyHV-2) [7,8], but larger than those of other members of the order *Herpesvirales* which generally range from 125 to 240 kbp. Phylogenetic analysis of the CyHV-3 genome sequence led to its classification in the new family *Alloherpesviridae* encompassing herpesviruses of fish and amphibians [9,10]. The CyHV-3 genome contains 155 potential protein-coding open reading frames (ORFs), some of which have relatives in other herpesviruses, and a few of which have relatives in poxviruses, iridoviruses and other large DNA viruses [6,8,11]. Interestingly, CyHV-3 genome encodes proteins potentially involved in immune evasion mechanisms such as, for example, TNF receptor homologues (encoded by ORF4 and ORF12) and an IL-10 homologue (encoded by ORF134) [6].

Cellular IL-10 has been described in a wide range of vertebrate species, including fish [12,13]. It is a pleiotropic immunomodulatory cytokine with both immunostimulating and immunosuppressive properties [14]; however, IL-10 is generally described as an immunosuppressive cytokine. It

* Correspondence: a.vdplasschen@ulg.ac.be
[1]Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases (B43b), Faculty of Veterinary Medicine, University of Liège, Liège B–4000, Belgium
Full list of author information is available at the end of the article

inhibits expression of a large number of cytokines as, for example, TNF-α, IFN-γ, IL-1β, IL-2, IL-3, IL-6, and MHC class II [15-17]. Many viruses exploit the immunosuppressive properties of IL-10 to evade immune recognition either by up-regulation of host IL-10 or by expression of virally encoded IL-10 homologues (vIL-10s) [14,18,19].

Virally encoded IL-10 homologues have been reported in members of the *Poxviridae* family and the *Herpesvirales* order [19-21]. Among the *Herpesvirales* order, vIL-10s have been described in members of the *Herpesviridae* (e.g. human cytomegalovirus [HCMV] and Epstein-Barr virus [EBV]) and more recently in the family *Alloherpesviridae* (Anguilid herpesvirus 1 [AngHV-1] and CyHV-3) [22]. While the role of vIL-10s has been demonstrated in the pathogenesis of one *Poxviridae* and one *Herpesviridae* [23-25]; this has not yet been investigated in the family *Alloherpesviridae*. However, a very recent study suggested that the IL-10 homologue encoded by CyHV-3 ORF134 could play a role in the pathogenesis. Firstly, it has been demonstrated that this ORF is transcribed in infected fish maintained at permissive and even restrictive temperature [26]. Secondly, it has been shown that injection of CyHV-3 ORF134 mRNA into zebrafish embryos increased the number of lysozyme-positive cells to a similar degree as zebrafish IL-10 [26]; an effect that was inhibited by down regulation of the IL-10 receptor long chain using a specific morpholino [26].

The present study was devoted to CyHV-3 ORF134 encoding an IL-10 homologue. In vitro studies demonstrated that ORF134 is expressed as a spliced early-late gene and that its expression product is the second most abundant viral protein in the CyHV-3 secretome. Taking advantage of the recent BAC cloning of CyHV-3 as an infectious bacterial artificial chromosome (BAC), a strain deleted for ORF134 and a derived revertant strain were produced. Comparison of these strains demonstrated that ORF134 is essential neither for CyHV-3 replication in vitro nor for virulence in common carp.

Materials and methods

Cells and viruses

Cyprinus carpio brain cells (CCB) were cultured in minimum essential medium (MEM) (Invitrogen, Merelbeke, Belgium) containing 4.5 g/L glucose (D-glucose monohydrate; Merck, Darmstadt, Germany) and 10% fetal calf serum (FCS). Cells were cultured at 25 °C in a humid atmosphere containing 5% CO_2. The CyHV-3 FL strain was isolated from the kidney of a fish that died from CyHV-3 infection (CER, Marloie, Belgium) [27].

Determination of ORF134 kinetic class of transcription

These experiments were performed as described elsewhere [28]. Briefly, monolayers of CCB cells in 24-well plates were pre-incubated for 2 h before infection with cycloheximide (CHX) (100 μg/mL) (Sigma-Aldrich, Saint Louis, Missouri, USA) or phosphonoacetic acid (PAA) (300 μg/mL) (Sigma-Aldrich), the inhibitors of de novo protein synthesis or viral DNA polymerase, respectively. After removal of the medium, cells were infected with CyHV-3 FL strain at a multiplicity of infection (MOI) of 0.1 plaque forming unit (PFU) per cell in presence of inhibitors. After an incubation of 2 h, cells were overlaid with fresh medium containing the inhibitors. At 6, 8 and 12 h after inoculation cells were harvested and treated for RT-PCR analysis of gene expression (see below). CyHV-3 ORF3 (immediate early [IE]), ORF55 (early [E]) and ORF78 (late [L]) were used as reference gene of the three kinetic classes [29,30].

Transcriptional analysis by RT-PCR

Cytoplasmic RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) with on column DNase I digestion. cDNA was synthetized from 1 μg of RNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Nazareth Eke, Belgium). Finally, PCRs were performed with the primers listed in Table 1 (see RT-PCR column).

Production of concentrated cell supernatant

CCB cells were infected with CyHV-3 FL strain at a MOI of 0.05 PFU per cell using serum free culture medium. Cell supernatants were collected 72 h post-inoculation and then submitted to two cycles of centrifugation at 4 °C (clarification at 2000 *g* for 15 min followed by pelleting of viral particles at 100 000 *g* for 2 h through a 30% sucrose gradient). The supernatant was then concentrated 25-fold by centrifugation (2000 *g*, 75 min, 4 °C) through an Amicon Ultra-15 centrifugal filter unit (3K NMWL; Merck Millipore, Billerica Massachusetts, USA) and stored at −80 °C until use.

2D-LC MS/MS proteomic approach

Proteomic analyses were performed using 2D-LC MS/MS workflow as described previously [31]. Briefly, proteins were reduced at 4 °C for 1 h with 10 mM DTT and alkylated by incubation with 25 mMiodoacetamide at 4 °C for 1 h in the dark. Proteins were recovered through acetone precipitation and digested with trypsin at an enzyme: substrate ratio of 1:50 in 50 mM NH_4HCO_3 overnight at 37 °C. Tryptic peptides (25 μg) were analysed by bidimensional (SCX-RP) chromatography and online MS/MS, as described elsewhere [32], except that only 3 successive salt plugs of 25, 100 and 800 mM NH_4Cl were used. Peptides were analyzed using the "peptide scan" option of an HCT ultra ion Trap (Bruker, Evere, Belgium), consisting of a full-scan mass spectrometry (MS) and MS/MS scan spectrum acquisitions in ultrascan mode (26 000 m/z sec-1). Peptide fragment mass spectra were acquired

Table 1 Primers and probes.

Targeted gene	Primer/probe name			Sequence (5'- 3')	Accession n°/ reference
Primers for PCR and RT-PCR		PCR	RT-PCR		
CyHV-3 ORF3	ORF3InF		●	TATGCCCACATGATGCTGTT	DQ657948
	ORF3InR		●	CAGTCAGACCCTTCCTCTGC	
CyHV-3 ORF55	ORF55InF	●		AGCGCTACACCGAAGAGTCC	
	ORF55stopR	●		TCACAGGATAGATATGTTACAAG	
	ORF55ATGF		●	ATGGCTATGCTGGAACTGG	
	ORF55InR		●	GGCGCACCCAGTAGATTATG	
CyHV-3 ORF78	ORF78InF		●	TGGACGACGAACACCCTTC	
	ORF78InR		●	GGTAGAGGGTACAACCACG	
CyHV-3 ORF132	ORF132InF		●	GGATCCGTTTTCTGGGTCTG	
	ORF132InR		●	CTCAATCCCTCACCGACCTC	
CyHV-3 ORF133	ORF133InF		●	GACGAGATCCCTATCCGCAG	
	ORF133InR		●	GACCTCGGGTATGGTCGGTA	
CyHV-3 ORF134	ORF134stopF		●	TCAATGTTTGCGCTTGGTTTTC	
	ORF134ATGR		●	ATGTTCCTTGCAGTGCTAC	
	ORF134InF	●		GGTTTCTCTTTGTAGTTTTCCG	
	ORF134InR	●		CACCCCAACTTTTGAGACAAC	
	ORF134outseqF	●		GTCAACATGGACGAGCGTGA	
	ORF134outseqR	●		GTGGGGATATCAAACACGCA	
CyHV-3 ORF135	ORF135InF		●	ACACCACCAACGAGACATGC	
	ORF135InR		●	CTTTTCGGACCAGAAGACCG	
Carp β-actin	Actin-F		●	ATGTACGTTGCCATCCAGGC	M24113
	Actin-R		●	GCACCTGAACCTCTCATTGC	
Primers for amplification of recombination cassettes					
H1-*gal*K-H2 cassette	134 *gal*K F			ATGTTCCTTGCAGTGCTACTAACCG	Warming et al. [34]
				CGACCATCTTCTTCGAGGCTCGGGG	
				CCTGTTGACAATTAATCATCGGCA	
	134 *gal*K R			TCAATGTTTGCGCTTGGTTTTCATG	
				TTCTTGACGTCTTTTGCGACCAGGA	
				TCAGCACTGTCCTGCTCCTT	
H1-ORF134-H2 cassette	H1F			GCTCATCAATCGCAGCAGCA	DQ657948
	H2R			CAAGCCATTATCCTGTTGGG	
Primers and probes for real-time TaqMan PCR quantification of CyHV-3 genome					
CyHV-3 ORF89	KHV-86F			GACGCCGGAGACCTTGTG	AF411803
	KHV-163R			CGGGTTCTTATTTTTGTCCTTGTT	
	KHV-109P			(6FAM)CTTCCTCTGCTCGGCGAGCACG(BHQ1)	
Carp glucokinase	CgGluc-162F			ACTGCGAGTGGAGACACATGAT	AF053332
	CgGluc-230R			TCAGGTGTGGAGCGGACAT	
	CgGluc-185P			(6FAM)AAGCCAGTGTCAAAATGCTGCCCACT(BHQ1)	
Primers for RT-qPCR analysis of carp gene expression					
40S	40S-F			CCGTGGGTGACATCGTTACA	AB012087
	40S-R			TCAGGACATTGAACCTCACTGTCT	

Table 1 Primers and probes. *(Continued)*

IL-1β	IL-1β-F	AAGGAGGCCAGTGGCTCTGT	AJ245635
	IL-1β-R	CCTGAAGAAGAGGAGGCTGTCA	
TNF-α1 and 2	TNF-α1 and 2-F	GCTGTCTGCTTCACGCTCAA	AJ311800
	TNF-α1 and 2-R	CCTTGGAAGTGACATTTGCTTTT	
CXCa	CXCa-F	CTGGGATTCCTGACCATTGGT	AJ421443
	CXCa-R	GTTGGCTCTCTGTTTCAATGCA	
IL-10	IL-10-F	CGCCAGCATAAAGAACTCGT	AB110780
	IL-10-R	TGCCAAATACTGCTCGATGT	
IFNγ-2	IFNγ-2-F	TCTTGAGGAACCTGAGCAGAA	AM168523
	IFNγ-2-R	TGTGCAAGTCTTTCCTTTGTAG	
IL-6	IL-6M17-F	CACATTGCTGTGAGGGTGAA	AY102633
	IL-6M17-R	GCATCCATAGGCTTTCTGCT	

Underlined: 50bp corresponding to CyHV-3 sequence.

in data-dependent AutoMS (2) mode with a scan range of 100–2,800 m/z, three averages, and 5 precursor ions selected from the MS scan 300–1500 m/z. Precursors were actively excluded within a 0.5 min window, and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using Data Analysis 2.4 software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS Ions searches of the NCBInr database release 20120809 using an in-house Mascot 2.2 server (Matrix Science). The default search parameters used were: Taxonomy = Bony vertebrates or Cyprinivirus; Enzyme = Trypsin; Maximum missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.2 Dalton (Da); MS/MS tolerance ± 0.6 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP. All data were also searched against the NCBI bony vertebrate database in order to detect host proteins. Only proteins identified with p value lower than 0.05 were considered, and single peptide identifications were systematically evaluated manually. In addition, the emPAI [33] was calculated to estimate protein relative abundance in the culture supernatant.

Production of CyHV-3 ORF134 recombinants

CyHV-3 recombinants were produced using prokaryotic recombination technologies (Figure 1). The FL BAC plasmid was used as parental plasmid [27]. In this plasmid, the BAC cassette is inserted in ORF55 encoding thymidine kinase (TK). ORF134 recombinant plasmids were produced using two-steps galactokinase gene (*gal*K) positive/negative selection in bacteria as described previously [34]. The first recombination process (*gal*K positive selection) consisted to replace ORF134 by *gal*K resulting in the FL BAC ORF134 Del *gal*K plasmid. Recombination was achieved using the H1-*gal*K-H2 recombination cassette (Figure 1b) which consisted of the *gal*K gene flanked by 50-bp sequences homologous to CyHV-3 genome regions flanking ORF134 deletion (Figure 1a). H1-*gal*K-H2 recombination cassette was produced by PCR (primers 134 *gal*K F and 134 *gal*K R) using the p*gal*K vector as template. Primer 134 *gal*K F consisted of nucleotides 229836–229885 (50bp) of CyHV-3 genome and 1–24 (24bp) of the p*gal*K vector. Primer 134 *gal*K R consisted of nucleotides 229262–229311 (50bp) of the CyHV-3 genome and nucleotides 1212–1231 (20bp) of the pgalK vector (Table 1). The 50-bp sequences of the H1-*gal*K-H2 corresponding to CyHV-3 genome were used to target homologous recombination in bacteria. The second recombination process (*gal*K negative selection) consisted to remove the *gal*K gene (FL BAC ORF134 Del plasmid) or to replace the *gal*K gene by CyHV-3 wild type ORF134 sequence (FL BAC ORF134 Rev plasmid) (Figure 1). The FL BAC ORF134 Del plasmid was obtained by recombination with the H1-H2 cassette (Figure 1b). This cassette was synthesized and consisted of 200 bp of CyHV-3 genome upstream and downstream of ORF134 deletion, respectively. The FL BAC ORF134 Rev plasmid was produced by recombination with the H1-ORF134-H2 cassette. This cassette was produced by PCR (primers H1F and H2R) using CyHV-3 FL DNA as template corresponding to nucleotides 229057–229076 and nucleotides 230056–230075 of CyHV-3 genome, respectively. To reconstitute infectious virus encoding a wild type TK locus (removal of the BAC cassette), the BAC plasmids (FL BAC, FL BAC ORF134 Del and FL BAC ORF134 Rev) were co-transfected with the pGEMT-TK plasmid (molecular ratio, 1:75) into CCB cells [27]. Plaque negative for enhanced green fluorescent protein (EGFP) expression (the BAC cassette encodes an EGFP expression cassette) were picked and amplified.

Southern blotting

Southern blot analysis of recombinant viruses was performed as described previously [27,35]. PCRs were

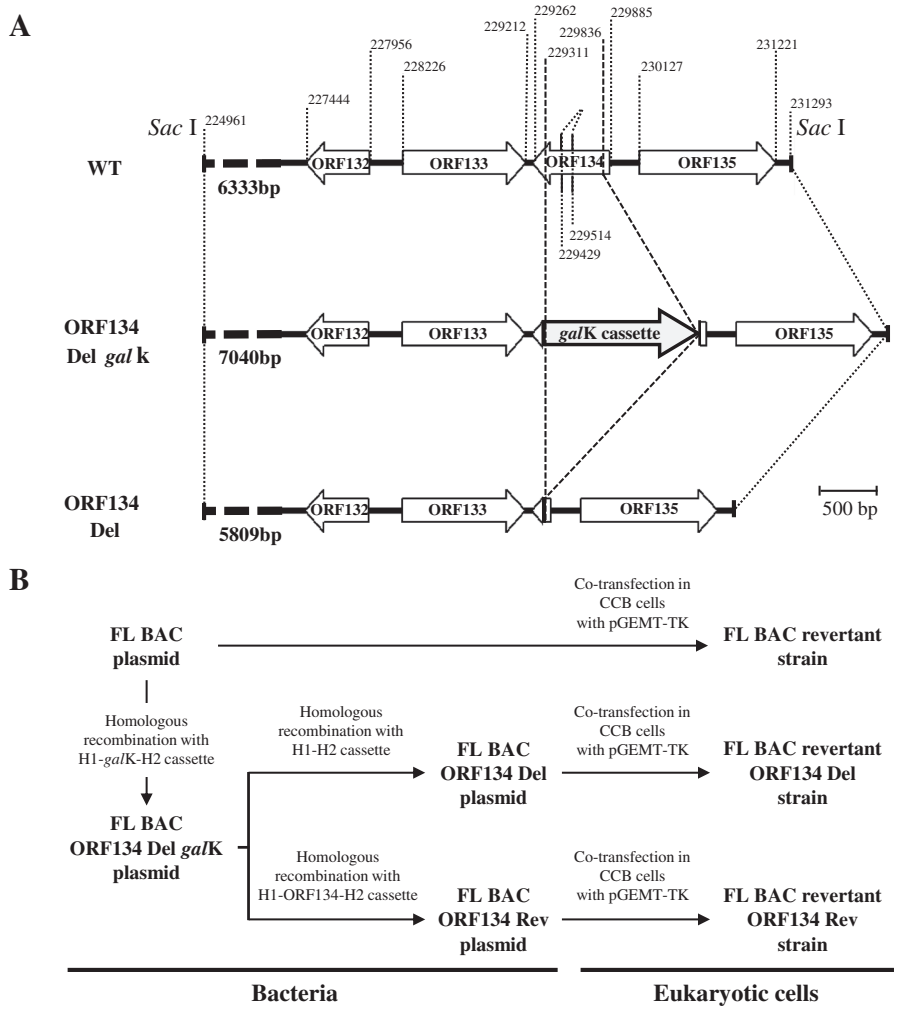


Figure 1 Schematic representation of the strategy used to produce CyHV-3 FL BAC ORF134 recombinants. (**A**) The region of CyHV-3 genome encoding ORF134 is illustrated for wild type (WT), ORF134 Del *gal*K and ORF134 Del genotypes. (**B**) Flow chart of stages performed to produce FL BAC ORF134 recombinant plasmids and to reconstitute virus strains.

performed to produce ORF55 probe (primers ORF55InF and ORF55stopR) and ORF134Del probe (primers ORF134InF and ORF134InR) using the CyHV-3 FL genome as a template (Table 1).

Multi-step growth curves

Triplicate cultures of CCB cells were infected at a MOI of 0.5 PFU per cell. After an incubation period of 2 h, cells were washed with phosphate-buffered saline (PBS) and then overlaid with Dulbecco's modified essential medium (DMEM, Invitrogen) containing 4.5 g of glucose/liter and 10% FCS. Supernatant of infected cultures was harvested at successive intervals after infection and stored at −80 °C. The amount of infectious virus was determined by plaque assay on CCB cells as described previously [35].

Fish

Common carp (*Cyprinus carpio carpio*) (CEFRA, University of Liège, Belgium), were kept in 60-liter tanks at 24 °C. Microbiological, parasitical and clinical examinations of the fish just before the experiments demonstrated that these fish were fully healthy.

CyHV-3 inoculation of carp

For viral inoculation mimicking natural infection, fish were kept for 2 h in water containing CyHV-3. At the end of the incubation period, fish were returned to larger tanks. In some experiments, fish that survived the primary infection were challenged 42 days after inoculation by cohabitation with fish that were infected by immersion in water containing 200 PFU/mL of the FL strain just before their release into the tank to be challenged. Two freshly infected fish were released per tank to be challenged. The

animal study was accredited by the local ethics committee of the University of Liège, Belgium (Laboratory accreditation N°1610008, protocol N°810).

Quantification of virus genome copies in organs by real-time TaqMan PCR

Virus genome quantitation was performed by real-time TaqMan PCR as described elsewhere [36]. The primers and the probes used are presented in Table 1. Two sets of primers were used to amplify fragments of CyHV-3 ORF89 and carp glucokinase genes. The amplicons were cloned into the pGEM-T Easy vector and the resulting plasmids were used to generate standard curves by running reactions with 10^1 to 10^{10} plasmid molecules. DNA was isolated using a DNA mini kit (Qiagen) from 25 mg of organs stored at −80 °C in RNAlater® (Invitrogen). The reaction mix contained 1 × iQSupermix (Bio-Rad), 200 nM of each primer, 400 nM of fluorescent probe and 250 ng of DNA. The analyses were performed using a C1000 Touch Thermal cycler (Bio-Rad). All real-time TaqMan PCRs for CyHV-3 DNA were run with equal amounts of DNA estimated by the real-time TaqMan PCR performed on carp glucokinase gene.

Quantification of carp gene expression in spleen by RT-qPCR

Total RNA was isolated from spleens stored at −80 °C in RNALater® (Ambion®, Invitrogen, Merelbeke, Belgium) using TRI reagent® (Ambion®, Invitrogen), including DNase I digestion and RNA purification using RNeasyMinElute Cleanup Kit (Qiagen). cDNA was synthetized from 1 μg of RNA using iScriptcDNA Synthesis Kit (Bio-Rad). The primers used for RT-qPCR were described previously [37] and are listed in Table 1. The RT-qPCR master-mix was prepared as follows: 1 × IQ™ SYBR® Green Supermix (Bio-Rad), 200 nM of each primer, 5 μL of 25 × diluted cDNA and sterile water to a final volume of 25 μL. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 40 cycles with denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s. At the end, the dissociation stage was performed (95 °C for for 10 s) and the melt curve was obtained by increasing the temperature from 60 °C to 95 °C with a rate of 0.5 °C per 5 s. Fluorescence data from RT-qPCR experiments were analyzed using the CFX96 real-time system and exported to Microsoft Excel. The threshold cycle (Ct) was determined using the Auto method for all runs. The expression of analyzed genes was calculated using the $2^{-\Delta\Delta Ct}$ method [38]. The 40S ribosomal protein S11 was used as a reference gene.

Histological analysis

Organs from mock-infected or infected carp were fixed in 4% buffered formalin and embedded in paraffin blocks. Sections of 5 μm were stained with haematoxylin and eosin prior to microscopic analysis [39].

Statistical analyses

Multi-step growth curves data expressed as mean titer ± standard deviation (SD) were analyzed for significance of differences ($p< 0.05$) using one-way ANOVA. The differences in mortality induced by the CyHV-3 strains tested were analyzed using Kaplan and Meier survival analysis. Significant differences ($p< 0.05$) in virus load between fish infected with the different CyHV-3 strains at each sampling point were assessed using one-way ANOVA followed by Holm-Sidak test when data were normally distributed, or with the non-parametric Kruskal–Wallis test followed by Tukey test when they were not. Significant differences ($p< 0.05$) in RT-qPCR gene expression between CyHV-3 infected and mock-infected fish, as well as between fish infected with different CyHV-3 strains at each sampling point were assessed using one-way ANOVA followed by Holm-Sidak test in cases where the data were normally distributed, or with the non-parametric Kruskal–Wallis test followed by Dunn's test when they were not

Results

CyHV-3 ORF134 kinetic class of expression

Two independent studies have demonstrated that CyHV-3 ORF134 is transcribed during viral replication in vitro thereby meeting the criteria for being a gene [26,29]. It has been predicted to contain an 84 bp intron flanked by 2 exons encoding a 179 amino acid product (GenBank accession number DQ657948). Here, we used CHX and PAA to identify the transcriptional class of ORF134 (Figure 2). This experiment revealed that ORF134 expression is prevented by CHX and reduced but not prevented by PAA treatments, suggesting that ORF134 is an E-L gene. ORF3, ORF55 and ORF78 were used as controls in this experiment; the results presented in Figure 2 confirmed that they are IE, E and L genes, respectively. The absence of contaminant viral DNA in the mRNA preparations was confirmed by the absence of a PCR product when the reverse transcriptase was omitted from the reactions. Furthermore, the estimated molecular size of the major ORF134 RT-PCR product revealed that it was derived, from the amplification of cDNA (540 bp) rather than from the viral genome (624 bp). This observation is consistent with the earlier description of the ORF134 as a spliced gene [40,41]. However, a minor product corresponding to the unspliced transcript of ORF134 was also observed (see the faint 624 bp band in Figure 2). The classification of ORF134 as an E-L gene is consistent with the results published recently by Ilouze et al. who concluded that ORF134 is

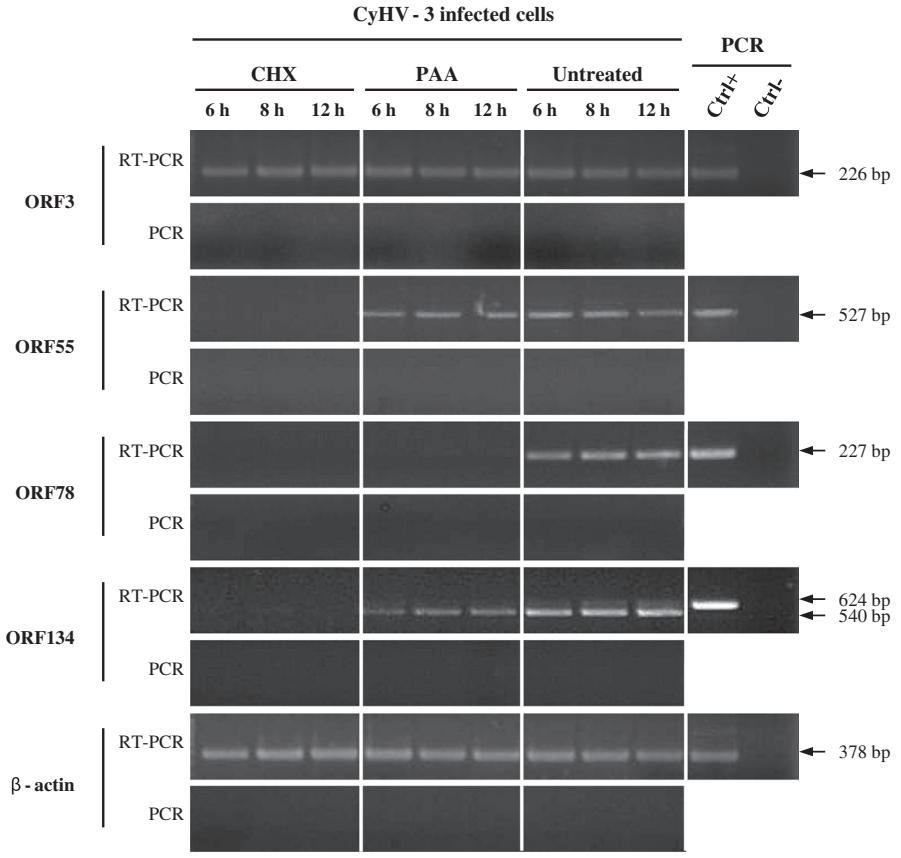


Figure 2 Determination of ORF134 kinetic class of transcription. CCB cells were infected with CyHV-3 FL strain, in absence (Untreated) or presence of CHX or PAA as described in Materials and methods. At the indicated time post-infection, expression of IE ORF3, E ORF55, L ORF78, ORF134 and carp β-actin was studied by a RT-PCR approach. On the left RT-PCR or PCR represent PCR products generated when the RT was performed or omitted from the reactions, respectively. On the right, control PCR reactions were performed using genomic DNA as template (Ctrl+) or no template (Ctrl-).

an E gene [29]. It is also consistent with the E expression reported for other vIL-10s [40,41].

CyHV-3 secretome

While two independent studies have previously shown that ORF134 is transcribed during viral replication [26,29], it is still to be determined whether ORF134 encodes a protein secreted from infected cells. To address this question, concentrated supernatant was produced from CyHV-3 infected CCB cultures and analyzed by 2D-LC MS/MS. Viral and cellular proteins identified by this approach are listed in Table 2. This list was restricted to proteins identified with p value lower than 0.05 as determined by the MASCOT program. Five viral and 46 cellular proteins were detected. CyHV-3 ORF12 and ORF134 were amongst the most abundant proteins in the sample as revealed by their relatively high emPAI scores (1.49 and 1.02, respectively). Only two cellular proteins had comparable scores (Beta-2-microglobulin and FK506 binding protein 1A, with emPAI scores of 1.79 and 1.39, respectively). ORF12 encodes a soluble TNF receptor superfamily homologue which, like ORF134, was expected to be secreted from infected cells. Three unique peptides covering 16% of the ORF134 sequence were sequenced (Figure 3a). These peptides were distributed throughout ORF134 sequence (Figure 3b). The divergence existing between CyHV-3 IL-10 and carp IL-10 excludes the hypothesis that the peptides detected could be derived from carp IL-10 rather than from CyHV-3 ORF134. In addition to CyHV-3 ORF12 and ORF134, three additional viral proteins (ORF52, ORF116 and ORF119) were detected in the CyHV-3 secretome. All three proteins are potential membrane proteins (Table 2). The presence of these putative membrane proteins in the CyHV-3 secretome cannot be explained by remaining viral particles in the prepared concentrated extracellular medium, as none of these proteins is structural [31]. It is also unlikely that the presence of these proteins reflects cell lysis resulting from the viral infection. Indeed, in such a case, a higher number of viral proteins would be expected, in

Table 2 CyHV-3 and host proteins identified by 2D-LC MS/MS in the supernatant of CyHV-3 infected CCB cells.

Database	Accession number	Description	Predicted MW (kDa)	Mascot score	No. of matching spectra	emPAI
		CyHV-3 proteins				
Cyprinivirus	gi|129560530	ORF12, TNF receptor superfamily homologue	19.2	671	14	1.49
	gi|129560652	ORF134, Interleukin 10 homologue	14.8	304	6	1.02
	gi|84181525	ORF116, predicted membrane glycoprotein	30.4	185	4	0.26
	gi|84181523	ORF119, putative uncharacterized protein containing an hydrophobic region	15.5	94	2	0.25
	gi|129560569	ORF52, predicted membrane glycoprotein	39.2	44	1	0.10
		Host proteins (species origin)				
Bony vertebrates	gi|122891218	Novel protein (zgc:103659) (*Danio rerio*)	51.7	410	13	0.42
	gi|136429	Trypsin (*Sus scrofa*)	25.1	281	9	0.53
	gi|297262447	Predicted keratin, type II cytoskeletal 1-like isoform 6 (*Macaca mulatta*)	65.3	268	3	0.12
	gi|37590349	Enolase 1, alpha (*D. rerio*)	47.4	255	8	0.35
	gi|326670662	Predicted collagen alpha-3(VI) chain-like (*D. rerio*)	11.3	227	8	0.17
	gi|51949771	Fibronectin 1b (*D. rerio*)	279.3	213	8	0.08
	gi|52218922	Pigment epithelium-derived factor precursor (*D. rerio*)	45.0	158	3	0.17
	gi|416696	Beta-2-microglobulin (*Cyprinus carpio*)	13.5	152	8	1.79
	gi|1351907	Serum albumin (*Bos taurus*)	71.2	149	11	0.36
	gi|395744345	Predicted keratin, type II cytoskeletal 1 (*Pongo abelii*)	25.8	139	3	0.15
	gi|229552	Albumin (*B. taurus*)	68.1	136	10	0.38
	gi|63102189	Pgd protein (D. rerio)	53.7	134	4	0.14
	gi|15718387	Gelatinase (*Paralichthys olivaceus*)	75.5	125	5	0.15
	gi|1703244	Fructose-bisphosphate aldolase C (*Carassius auratus*)	39.8	124	4	0.20
	gi|169154447	Fibronectin 1 (*D. rerio*)	275.6	117	5	0.05
	gi|15149946	Procollagen type I alpha 1 chain (*D. rerio*)	49.4	117	5	0.34
	gi|148726027	Cadherin 11, osteoblast (*D. rerio*)	88.9	112	5	0.13
	gi|4885063	Fructose-bisphosphate aldolase C (*Homo sapiens*)	39.8	107	2	0.20
	gi|28336	Mutant beta-actin (beta'-actin) (*H. sapiens*)	42.1	105	2	0.09
	gi|28317	Unnamed protein product (*H. sapiens*)	59.7	104	3	0.13
	gi|337758	Pre-serum amyloid P component (*H. sapiens*)	25.5	100	3	0.32
	gi|223582	Histone H4 (*H. sapiens*)	11.2	99	5	0.84
	gi|47971186	Carp C1q-like molecule (*C. carpio*)	20.3	98	2	0.19
	gi|223061	Ubiquitin (*Salmo sp.*)	8.5	92	4	0.31
	gi|27806751	Alpha-2-HS-glycoprotein precursor (*B. taurus*)	39.2	90	4	0.32
	gi|47086029	Myristoylated alanine-rich C kinase substrate 2 (*D. rerio*)	21.0	86	2	0.18
	gi|2133885	N-cadherin precursor (*D. rerio*)	87.4	80	3	0.09
	gi|18859555	Wnt inhibitory factor 1 precursor (*D. rerio*)	43.2	79	3	0.09
	gi|34595971	Prion-like protein 1 (*C. carpio*)	55.6	75	2	0.14
	gi|208609649	Collagen type I alpha 3 (*C. auratus*)	137.7	75	1	0.03
	gi|47085905	14-3-3 protein beta/alpha-B (*D. rerio*)	27.5	75	3	0.47
	gi|6644111	Nucleoside diphosphate kinase-Z1 (*D. rerio*)	17.4	72	2	0.22
	gi|16974825	Chain A, Solution Structure Of Calcium-Calmodulin N-Terminal Domain (*H. sapiens*)	8.5	70	2	0.49
	gi|41152406	FK506 binding protein 1A, 12kDa (*D. rerio*)	11.8	69	3	1.39

Table 2 CyHV-3 and host proteins identified by 2D-LC MS/MS in the supernatant of CyHV-3 infected CCB cells. *(Continued)*

gi|189527793	Predicted neuroblast differentiation-associated protein AHNAK (*D. rerio*)	642.1	65	2	0.01
gi|37492	Alpha-tubulin (H. sapiens)	50.8	65	2	0.07
gi|33989505	Tissue inhibitor of metalloproteinase 2b (*D. rerio*)	24.7	64	2	0.15
gi|8176557	Heart fatty acid binding protein (*Anguilla japonica*)	15.3	61	1	0.26
gi|37181	Tissue inhibitor of metalloproteinases, Type-2 (*H. sapiens*)	21.4	59	2	0.18
gi|437972	Fibrillin-2 (*H. sapiens*)	334.8	59	1	0.01
gi|37367051	Osteopontin (*D. rerio*)	23.2	53	1	0.16
gi|45544646	Cold inducible RNA binding protein isoform 2 (*D. rerio*)	19.2	52	2	0.20
gi|51328294	Fstl1b protein (*D. rerio*)	39.6	50	1	0.09
gi|82245450	Triosephosphate isomerase B (*D. rerio*)	27.1	50	1	0.14
gi|34014734	Clusterin (*D. rerio*)	52.5	50	1	0.07
gi|47228578	Unnamed protein product (*Tetraodon nigroviridis*)	77.5	49	1	0.05

particular the most abundant ones [31]. Several viral proteins are expressed as two different forms, a membrane-anchored form and a secreted form, the latter generated by proteolytic cleavage of the former [42,43]. Further experiments are required to determine whether this phenomenon applies to the putative CyHV-3 membrane proteins detected in the secretome.

The MS data presented above demonstrate that CyHV-3 ORF134 encodes a protein that is abundantly secreted in the extracellular medium by infected cells. This observation is consistent with the hypothesis that ORF134 may be a functional IL-10 homologue playing a role in CyHV-3 pathogenesis [26].

Production and characterization of CyHV-3 ORF134 recombinant strains

In order to investigate subsequently the importance of ORF134 in virus replication in vitro and pathogenesis

A

Accession number	Description	Sequence coverage	Peptide sequence	Peptide mascot score
GI 129560652	CyHV-3 ORF134	16%	SEVDEIGDNLSR	80
			KSEVDEIGDNLSR	52
			DSCVYLIGQTPQLLR	50

B

```
CyHV-3 ORF134      -MFLAVLLTATIFFEARGAPATTPKDS---CVYLIGQTPQLLRQLRNAYQ
Cyprinus carpio IL-10 MVFSGVILSALVMFLLSDSAQCRRVDCKTDCCSFVEGFPVRLKELRSAYR
                    :* .*:*:* ::*   .:.     *.   *  ::   *  *::**.**:

CyHV-3 ORF134      AIIGADGSGVDEDDMPIYPSDVMNELASTSVACDAIKKVLTMNIG-ILP-
Cyprinus carpio IL-10 EIQNFYES--NDDMEPLLDENVQQNINSP-YGCHVMNEILRFYLDTILPT
                    * .   *  ::*  *:  .:* ::: *.  .*..::::* : :. ***

CyHV-3 ORF134      NVTAAYPDKKSEVDEIGDNLSRLHQNIVNCRDFLKCD----LPHWHQMAE
Cyprinus carpio IL-10 AVQKDHLHSKTPINSIGNIFQDLKRDMRKCRNYFSCQNPLEIASIKNSYE
                    *   : ..*: ::.**: :. *:::: :**:::.*:    :.  ::  *

CyHV-3 ORF134      NYKEK-PMQGFSEMDFVFQSVEKFLVAKDVKNMKTKRKH
Cyprinus carpio IL-10 KMKEKGVSKAMGELDILFKYIEQYLASKRVKHL------
                    : ***   :.:.*:*::*: :*::*.:* **::
```

Figure 3 Identification of CyHV-3 ORF134 by 2D-LC MS/MS in the supernatant of CyHV-3 infected CCB cells. (**A**) Data collected on ORF134 through 2D-LC MS/MS analysis of cell culture supernatant. (**B**) Sequential alignment of CyHV-3 ORF134 and *Cyprinus carpio* IL-10. Sequence coverage: detected peptides are presented in rectangles.

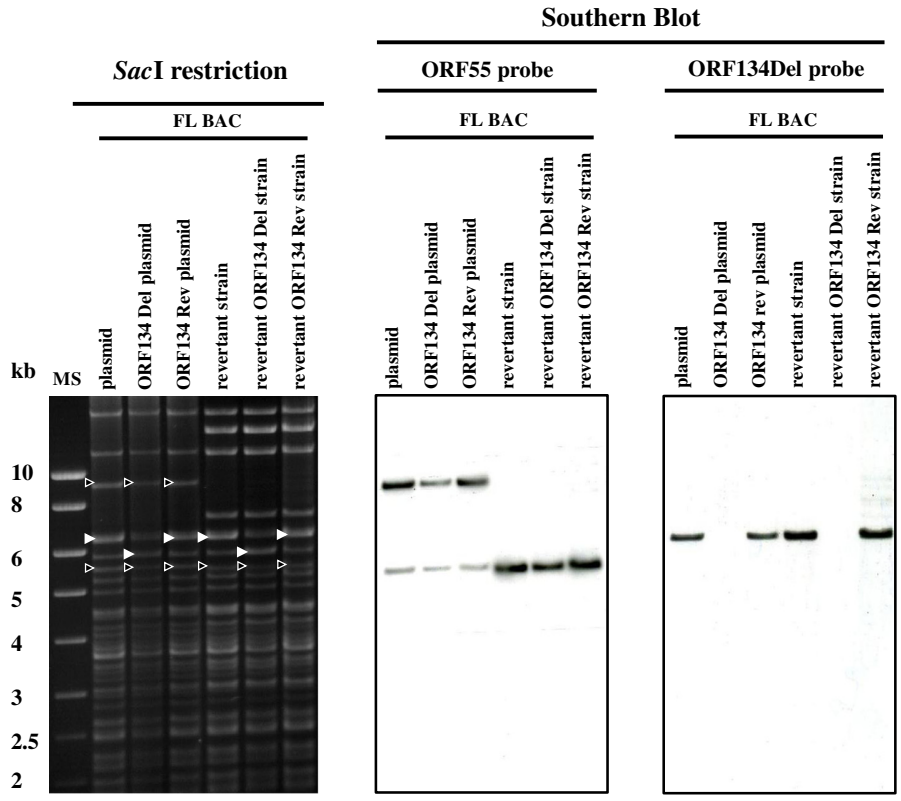


Figure 4 Structural analysis of the FL BAC ORF134 recombinant plasmids and derived CyHV-3 recombinant strains. The CyHV-3 FL BAC, FL BAC ORF134 Del and FL BAC ORF134 Rev plasmids and the genome of the FL BAC revertant, FL BAC revertant ORF134 Del and FL BAC revertant ORF134 Rev strains were analyzed by *Sac*I restriction (left panel) and further tested by southern blotting using probes corresponding to ORF55 (central panel) and ORF134 (right panel). White-outlined black arrowheads and white arrows indicate restriction fragments containing ORF55 and ORF134 loci, respectively. Marker sizes (MS) are indicated on the left.

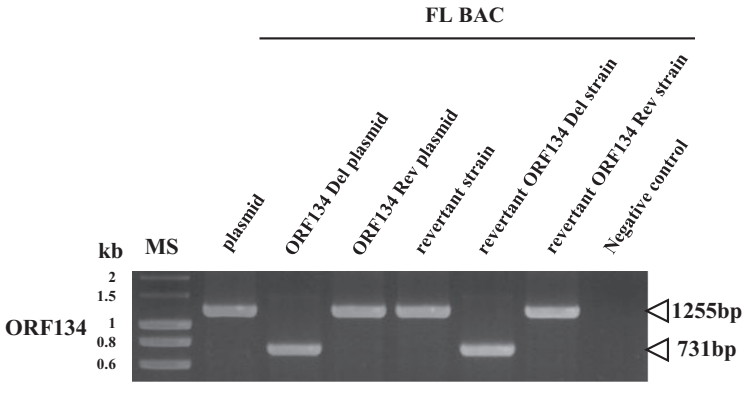


Figure 5 PCR analysis of the FL BAC ORF134 recombinant plasmids and derived CyHV-3 recombinant strains. The CyHV-3 FL BAC, FL BAC ORF134 Del and FL BAC ORF134 Rev plasmids and the genome of the FL BAC revertant, FL BAC revertant ORF134 Del and FL BAC revertant ORF134 Rev strains were analyzed by PCR using the forward primer ORF134outseqF and the reverse primer ORF134outseqR (Table 1). MS are indicated on the left.

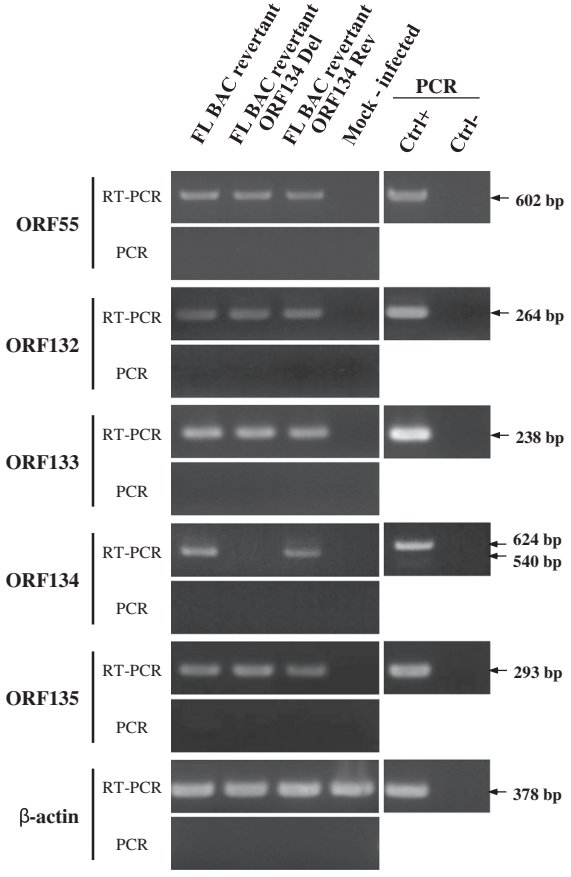


Figure 6 Transcriptional analysis of CyHV-3 ORF134 genome region. CCB cells were infected with the indicated recombinant strains at a MOI of 0.5 PFU/cell. Twenty-four hours post-infection, expression of CyHV-3 ORF55, ORF132, ORF133, ORF134, ORF135 and carp β-actin was studied by the RT-PCR approach described in the Materials and methods. On the left RT-PCR or PCR represent PCR products generated when the RT was performed or omitted from the reactions, respectively. On the right, control PCR reactions were performed using genomic DNA as template (Ctrl+) or no template (Ctrl-).

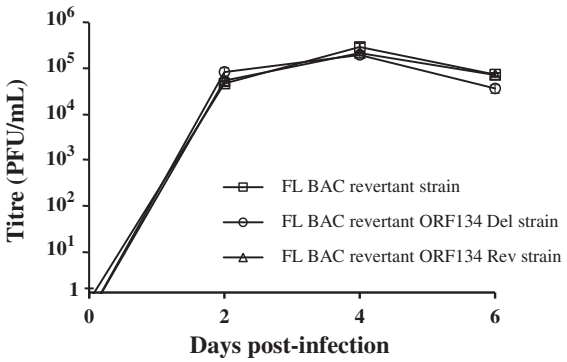


Figure 7 Effect of ORF134 deletion on viral growth in vitro. Replication kinetics of CyHV-3 ORF134 recombinant strains were compared with those of the FL BAC revertant strain using a multi-step growth assay (see Materials and methods). The data presented are the means ± standard errors of triplicate measurements.

in vivo, a CyHV-3 strain deleted for ORF134 (FL BAC revertant ORF134 Del strain) and a revertant strain (FL BAC revertant ORF134 Rev strain) were produced using BAC cloning and prokaryotic recombination technologies as described in the Materials and methods (Figure 1). The FL BAC plasmid was used as parental plasmid. A wild type strain (FL BAC revertant strain) was also reconstituted from the FL BAC plasmid. The molecular structures of the recombinant strains produced were confirmed by a combined *Sac*I restriction endonuclease and Southern blot approach targeting both ORF55 (the BAC cassette is inserted into the ORF55 locus) and ORF134 loci (Figure 4). In the three reconstituted strains, the ORF55 probe led to a single band corresponding to a 5.2 kb restriction fragment, demonstrating the reversion of ORF55 to wild type sequence (removal of the BAC cassette) [27]. In the FL BAC revertant and the FL BAC revertant ORF134 Rev, the ORF134Del probe led to a single band corresponding to a 6.3 kb restriction fragment consistent with the sequence of this region (6333kb). The absence of signal for the FL BAC revertant ORF134 Del demonstrated the deletion of ORF134. The molecular structure of the recombinants and the absence of contamination between strains was also controlled by PCR (Figure 5) and sequencing of the regions used to target recombination (data not shown). All approaches confirmed that the resulting recombinants have the correct molecular structure. Finally, using a RT-PCR approach, we controlled the process so that the deletion did not markedly affect the transcription of the ORFs located upstream and downstream of ORF134: ORF132, ORF133 and ORF135 (Figures 1a and 6). In these experiments, transcription of ORF55 was used as reference. For the three recombinants tested, transcripts of 602 bp, 264 bp, 238 bp and 293 bp were observed in infected cells for ORF55, ORF132, ORF133 and ORF135, respectively. No transcript was detected in mock-infected cells. When RT was omitted from the reactions, the product seen in infected cells was not detected, indicating that this product did not result from amplification of contaminant viral DNA. The three strains tested led to comparable signals for the four ORFs. Transcription analysis of ORF134 revealed that the FL BAC revertant and the FL BAC revertant ORF134 Rev expressed this ORF comparably, while no signal was detected for the FL BAC revertant ORF134 Del. Together, the results presented above demonstrate that the recombinants produced have the correct molecular structure and that the deletion of ORF134 has no marked polar effect on neighbor genes.

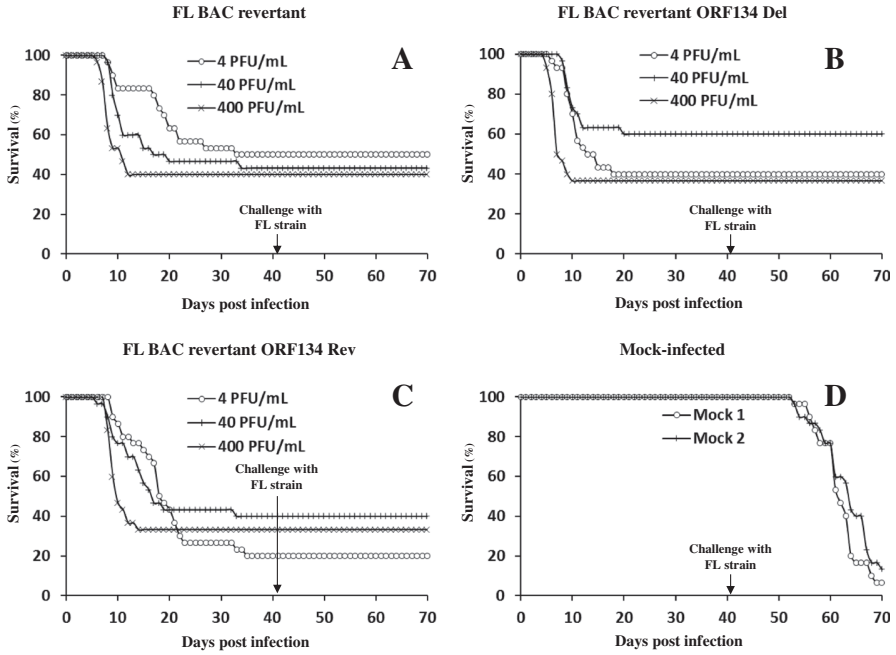


Figure 8 Cumulative survival rates of common carp infected with CyHV-3 recombinant strains. On day 0, common carp (n = 30 fish per tank), with an average weight of 3.8 g ± 1.5 g (mean ± SD), were mock-inoculated (2 tanks, panel **D**) or inoculated (panels **A**-**C**) by immersion for 2 h in water containing 4 PFU/mL, 40 PFU/mL or 400 PFU/mL of the indicated CyHV-3 strains. On day 42 post-infection (arrow), surviving fish were challenged by addition of two fish infected with the parental FL strain. Percentages of surviving carp are expressed according to days post-infection.

Effect of ORF134 deletion on viral growth in vitro

In order to investigate the putative effects of the recombination processes on viral growth in vitro, the FL BAC revertant, the FL BAC revertant ORF134 Del and the FL BAC revertant ORF134 Rev were compared using a multi-step growth assay (Figure 7). All viruses tested exhibited similar growth curves ($P \leq 0.05$), leading to the conclusion that ORF134 deletion does not affect viral growth in vitro (Figure 7). This observation is consistent with what has been reported for other vIL-10s [23,25]. Taken together, these results indicate that ORF134 is not essential for CyHV-3 replication in vitro and suggest that ORF134 exerts its biological functions in vivo.

Effect of ORF134 deletion on CyHV-3 pathogenesis

To investigate the importance of ORF134 in the pathogenesis of CyHV-3 disease, naïve common carp were inoculated by immersion in water containing the FL BAC

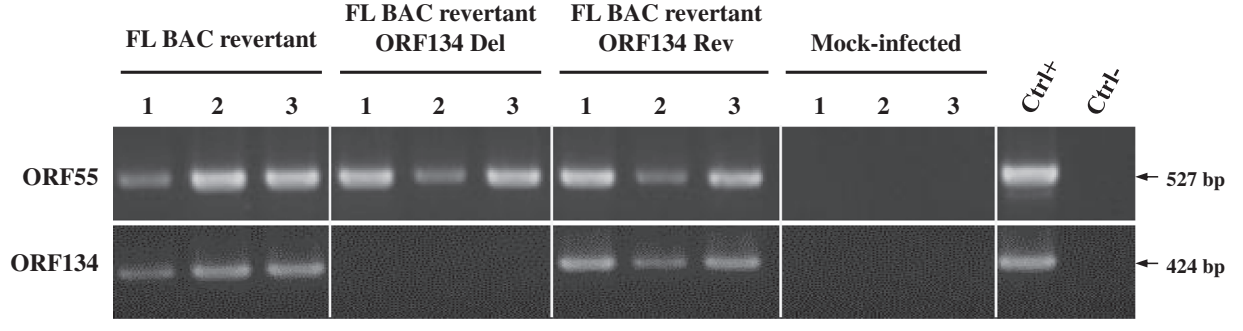


Figure 9 PCR detection and characterization of CyHV-3 genomes recovered from infected carp. The analyses reported in this figure are the follow-up of the experiment described in Figure 8. Three mock-infected carp (selected randomly before the challenge) and three dead carp from each of the groups infected with the FL BAC Revertant, FL BAC Revertant ORF134 Del and FL BAC Revertant ORF134 Rev strains were dissected. DNA was extracted from the kidney. PCRs were performed with the ORF55InF/ORF55stopR and ORF134InF/ORF134InR pairs of primers (Table 1). FL strain DNA and distilled water were used as positive (Ctrl+) and negative (Ctrl-) controls, respectively.

revertant, FL BAC revertant ORF134 Del or FL BAC revertant ORF134 Rev strains (Figure 8). The three strains induced at comparable levels all the clinical signs associated with the disease, including apathy, folding of the dorsal fin, hyperemia, increased mucus secretions, skin lesions, suffocation, erratic swimming, and the loss of equilibrium. The mortality rate and the kinetics of mortality observed for the three strains were not significantly different. At necropsy, similar lesions were observed for the three strains including the discoloration of gill filaments, herpetic skin lesions, and necrotic nephritis. To control that the infection of all groups of fish was performed with the correct viral strain and to exclude any possibility of wild type virus spread among tanks, PCR assays were performed on three randomly selected dead fish from each infected group and three mock-infected fish randomly selected (Figure 9). The PCR results confirmed that each tank was infected with the correct strain and demonstrated the absence of viral spread between tanks. Next, to determine whether the ORF134 deletion affects the adaptive immune response developed by fish that survived primary infection; surviving fish were challenged by co-habitation with fish inoculated with the wild type FL strain (Figure 8). Independently of the viral strain used for the primary infection, none of the challenged fish developed CyHV-3 disease (Figure 8). In contrast, CyHV-3 disease developed in the two tanks that were initially mock-infected. Taken together, the results presented above suggest that ORF134 deletion does not affect CyHV-3 pathogenicity in common carp and the protective immune response developed by surviving fish.

To further test these hypotheses, we investigated the effect of ORF134 deletion on viral load (Figure 10) and on cytokine expression (Figure 11) during CyHV-3 disease. Naïve common carp were inoculated by immersion in water containing the FL BAC revertant, FL BAC revertant ORF134 Del or FL BAC revertant ORF134 Rev strains (Figure 10). At different times after inoculation gill, kidney and spleen were collected from randomly selected fish. Viral loads were analyzed in gill and kidney by real-time TaqMan PCR (Figure 10) while cytokine expression was studied in spleen by RT-qPCR (Figure 11).

Real-time TaqMan PCR results demonstrated that fish infected with the three viral strains had statistically comparable viral loads in the gills and the kidney throughout the course of the experiment (Figure 10). Using the approach described in Figure 9, PCR reactions were performed on randomly selected fish demonstrated that each tank was infected with the correct strain and confirmed the absence of viral spread between tanks (data not shown). Together, these results suggested that ORF134 deletion has no effect on viral load during primary acute infection.

The spleen is one of the organs in which CyHV-3 is the most abundant during the course of acute infection [36]. It is also considered as one of the major lymphoid organ in teleost [44]. In order to study the effect of CyHV-3 ORF134 on the carp immune response, the kinetics of gene expression of the cytokines IFN-γ2, TNFα1, TNFα2, IL-1β, IL-6, CXCa and IL-10 were analyzed in spleen from fish infected with FL BAC revertant, FL BAC revertant ORF134 deleted and FL BAC revertant ORF134 Rev strains (Figure 11). Samples were collected over a period of 2 to 8 days post-infection and analyzed by RT-qPCR. The kinetics of expression of studied cytokines showed similar patterns to those observed previously [37]. Taking mock-infected fish as a reference, expression of several cytokines (IFN-γ2, IL-1β, IL-6, and IL-10) was up-regulated as early as day 3 post-infection. The most pronounced up-regulation was

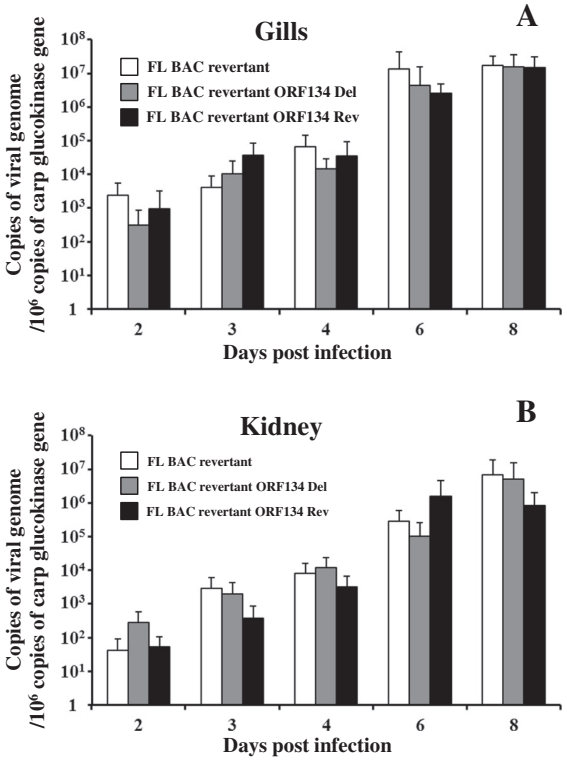


Figure 10 Viral load in gill and kidney. Common carp (n = 36 fish per tank), with an average weight of 10.7 g ± 3.2 g (mean ± SD), were inoculated by immersion for 2 h in water containing 100 PFU/mL of the indicated CyHV-3 strains. At different times post-inoculation, six infected fish were randomly selected per tank, euthanized and dissected. Six mock-infected fish were used as negative controls. Gill, kidney and spleen were harvested and stored in RNAlater® at −80 °C. DNA was extracted from gill (panel **A**) and kidney (panel **B**) and analyzed by real-time TaqMan PCR for quantification of viral genome copies. The results are expressed as the means ± SD of the data observed for the 6 fish analyzed per time point. Spleen were treated for quantification of carp gene expression by RT-qPCR (see Figure 11).

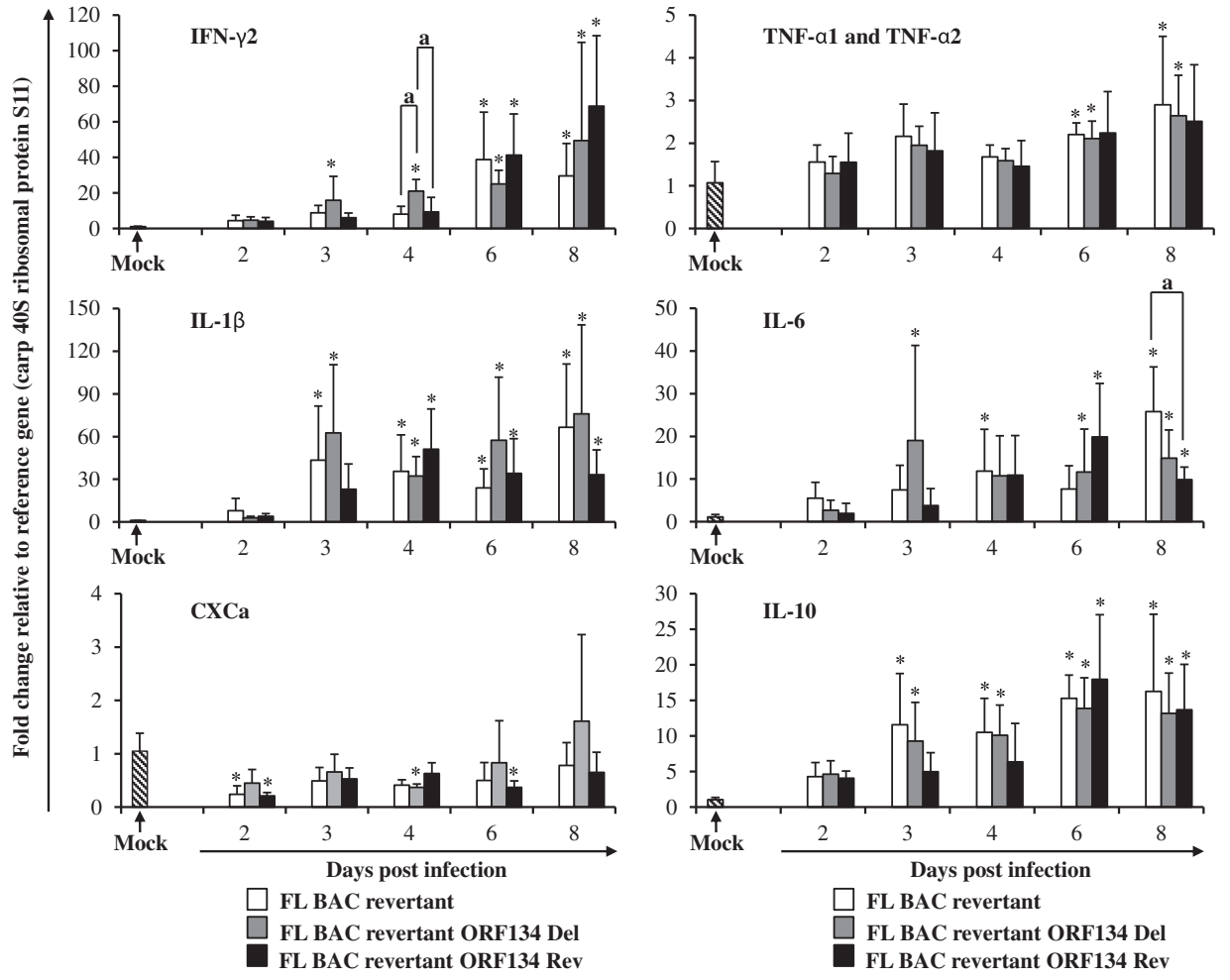


Figure 11 RT-qPCR analysis of cytokines. Kinetics of gene expression was measured in the spleen of mock-infected fish and fish infected with CyHV-3 ORF134 recombinants (see legend of Figure 10). Gene expression was normalized relative to the expression of the S11 protein of the 40S ribosomal subunit. Data are presented as mean ± SD ($n = 6$). Symbol (*) indicates statistical differences ($p \leq 0.05$) observed between infected and mock-infected fish. Symbol (a) indicates statistical differences ($p \leq 0.05$) observed for a specific time point between groups of fish infected with different CyHV-3 recombinants.

observed for IL-1β and IFN-γ2. We observed a moderate and late (day 6 and day 8 post-infection) up-regulation of TNFα1 and TNFα2. The expression level of CXCa in infected fish was comparable to mock-infected fish or even down-regulated for some strains at some time points. Importantly, the results presented in Figure 11 demonstrate that there is almost no difference in the expression levels of the cytokines studied between carp infected with three virus strains. The only significant differences observed between virus strains were for IFN-γ2 at day 4 post-inoculation and for IL-6 at day 8 post-inoculation. The expression level of IFN-γ2 at day 4 post-inoculation was significantly higher in fish infected with FL BAC revertant ORF134 deleted as compared to FL BAC revertant and FL BAC revertant ORF134 Rev strains. However, this difference was rather small and was not observed for the other sampling points, suggesting that it could reflect data variation rather than the expression of ORF134 biological activities. Supporting the latter hypothesis, the expression level of IL-6 at day 8 post-inoculation was significantly higher in the FL BAC revertant group as compared to the FL BAC revertant ORF134 Rev group. The absence of cross-contamination between tanks was controlled using the approach described in Figure 9 (data not shown). Together, these results suggested that ORF134 does not significantly affect the carp immune response under the experimental conditions used.

Finally, to investigate further the effect of ORF134 in CyHV-3 pathogenesis, the lesions induced by the FL BAC revertant, FL BAC revertant ORF134 Del and FL

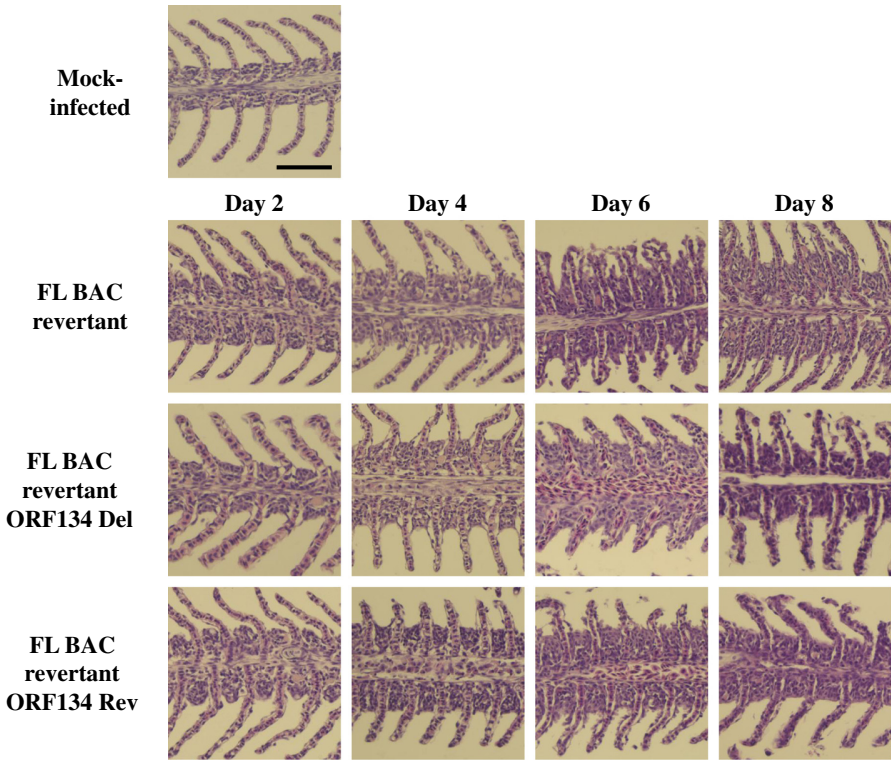


Figure 12 Histopathological characterization of the lesions induced by CyHV-3 ORF134 recombinants in the gills. Common carp (n = 20 fish per tank), with an average weight of 6 g ± 1.6 g (mean ± SD), were inoculated by immersion for 2 h in water containing 40 PFU/mL of the FL BAC revertant, FL BAC revertant ORF134 Del or FL BAC revertant ORF134 Rev strains. At different times post-inoculation (2, 4, 6 and 8 days), three infected fish were randomly selected per tank, euthanized and dissected. Three mock-infected fish were used as negative controls. Gills (present figure) and kidney (see Figure 13) were collected and processed for histological examination. Slides corresponding to each sampling point were grouped according to the viral strain used for the inoculation and were submitted to histopathological examinations by two independent examiners using a double-blind test mode. The images in this figure are representative of the analysis of one selected fish per group. Bar, 30 μm.

BAC revertant ORF134 Rev strains were compared in the gills and the kidney at various time points after infection (Figures 12 and 13). Histopathological preparations were grouped according to the virus genotype used for the infection and the time point of sampling. The groups of slides were observed by two independent examiners using a double-blind test. The principal histopathological changes were observed in gill filaments. Gills from mock-infected fish exhibited a normal structure. However, a weak lymphocytic hyperplasia was observed for the three mock-infected fish at the basis of the secondary lamellae, leading to their fusion. Few eosinophilic granulocytes were also observed along the primary lamella. As early as 2 days post-infection, both examiners were able to discriminate the three groups of infected fish from the mock-infected group. For all three infected groups, we observed congestion of the secondary lamellae, infiltration of lymphocytes and histiocytes at the basis of secondary lamellae further increasing their fusion. With the exception of one fish from the FL BAC revertant ORF134 Del group that exhibited weaker histopathological changes (see Figure 12, Day 2), the two other fish from this group expressed changes comparable to those observed in the two other infected groups. The absence of differences between the three viral groups was confirmed at the latter time points. At day 4 post-infection, all fish expressed comparable increased lymphocytic and histocytic infiltrate at the basis of the secondary lamellae. In some fish, an increase of eosinophilic granulocytes was observed (FL BAC revertant: 2 out of 3 fish; FL BAC revertant ORF134 Del: 2 out of 3 fish and FL BAC revertant ORF134 Rev strains 1 out of 3 fish). In comparison to day 2 post-infection, the infiltrate was more pronounced and the congestion was associated with edema of the secondary lamellae. The intensity of the lesions increased comparably in all three groups at latter time-points (Day 6 and Day 8). The infiltrate mainly lymphocytic induced the fusion of the lamellae on approximately 2/3 of their length. The respiratory epithelium exhibited hyperplasia and necrosis, associated in few cells with intranuclear inclusion

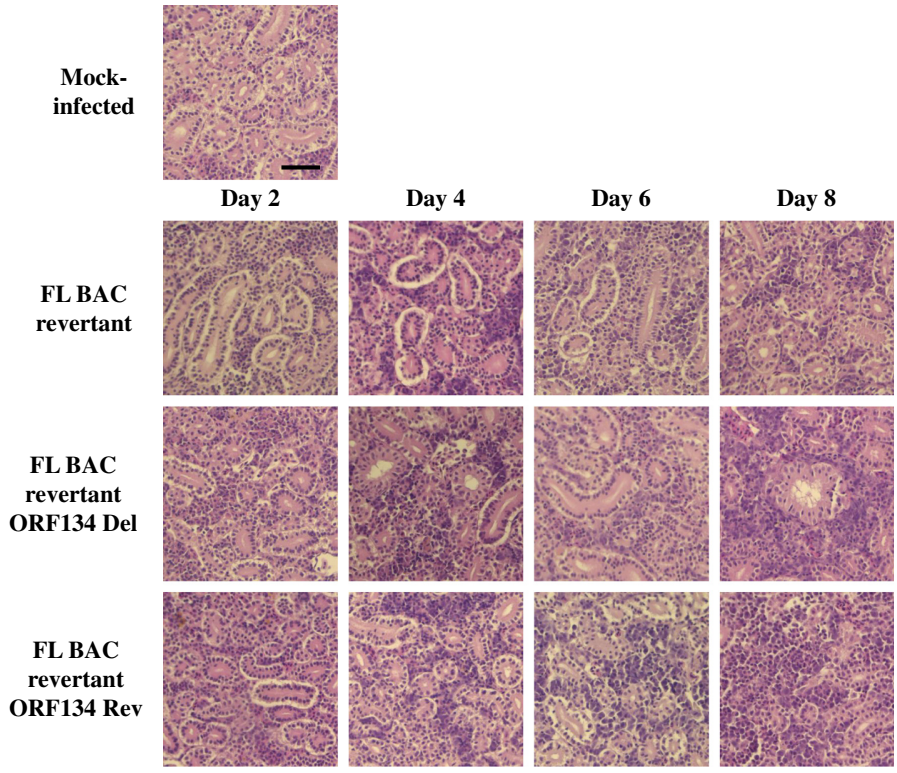


Figure 13 Histopathological characterization of the lesions induced by CyHV-3 ORF134 recombinants in the kidney. The fish infection methods are described in the legend of Figure 12.

bodies. Compared to day 6 post-infection, the infiltrate observed on day 8 was slightly reduced while the edema and the necrosis were increased. The lesions induced by the three recombinant strains were also compared in the kidney (Figure 13). The lesions observed in this organ were less obvious than in the gills. On day 2 post-infection, infected groups could not be differentiated from the mock-infected one. The diversity and the abundance of hematopoietic cells were normal. However, a slight increase of eosinophilic cells was observed in nearly all groups. Vacuolization of the epithelium was observed in all preparations, and was considered to be a preparation artifact. Starting on day 4 post-infection, both examiners were able to discriminate the three infected groups from the mock-infected one. However, they could not differentiate the three infected groups. Comparable proliferation of the hematopoietic cells, mainly lymphocytic and eosinophilic, was observed in all infected groups. The proliferation increased further on day 6 and 8. Intranuclear inclusion bodies were observed in a few hematopoietic cells on days 6 and 8, and in few epithelial cells on day 8. The absence of cross-contamination between tanks was controlled using the approach described in Figure 9 (data not shown).

Discussion

The present study was devoted to CyHV-3 ORF134, which encodes a potential vIL-10. We confirmed that ORF134 is transcribed as a spliced E-L gene (Figure 2). We also demonstrated for the first time that it is one of the most abundant proteins of the CyHV-3 secretome (Table 1) and that ORF134 is essential neither for viral replication in vitro nor for virulence in vivo. The latter conclusion relied on the observations that an ORF134 deleted strain could not be differentiated from its parental and revertant strains based on induced clinical signs and mortality rate (Figure 8), kinetic of viral load in gills and kidney (Figure 10), kinetic of cytokine expression in the spleen (Figure 11) and histological examination of gill and kidney (Figure 12).

As described in the introduction, cellular IL-10 is a pleiotropic immunomodulatory cytokine with both immunostimulatory and immunosuppressive properties [14]. Virally encoded IL-10 homologues have been reported in several members of the *Poxviridae* family and the *Herpesvirales* order [19-21]. Numerous molecular and in vitro studies suggest that there has been adaptive evolution of viral IL-10 following capture through positive selection to retain properties most beneficial for

the virus life cycle. However, very few studies have addressed the role of viral IL-10 in vivo by comparison of a wild type strain and derived deleted and revertant strains. This approach, which is the only one that can test the in vivo biological relevance of a gene, has been performed for only two viruses: rhesus cytomegalovirus (rhesus CMV) and Orf virus (ORFV) [23-25]. For both viruses, deletion of viral IL-10 induced virus attenuation and modulation of the host anti-viral innate immune response.

The results of the present study demonstrate that the IL-10 homologue encoded by CyHV-3 does not affect significantly its virulence in common carp (Figure 8) or the host innate immune response (Figure 11). However, a recent study based on an in vivo artificial model suggested that CyHV-3 ORF134 encodes a functional vIL-10 [26]. As IL-10 is known to induce a transient neutrophilia and monocytosis in addition to T cell suppression [45], these authors tested the in vivo functionality of CyHV-3 encoded IL-10 by injection of zebrafish embryos with mRNA encoding CyHV-3 ORF134 and analysis by whole-mount *in situ* hybridization (using a pan-leukocyte marker lysozyme at 56 hours post-fertilization before development of T cells). A slight but statistically significant increase in the number of lysozyme positive cells was observed in embryos injected with CyHV-3 ORF134 mRNA compared to control embryos. The effect observed was inhibited by down regulation of the IL-10 receptor long chain by a specific morpholino. These data suggested that CyHV-3 ORF134 encodes a functional vIL-10. Importantly, the ORF134 sequence used in this study is identical to the sequence encoded by the CyHV-3 strain used in the present study. Various hypotheses could explain the apparent paradox between the functional effect reported by Sunarto et al. and the lack of effect of deleting ORF134 described in the present study [26].

Firstly, it is possible that the slight effect observed by Sunarto et al. using optimal artificial conditions (overexpression of ORF134, no inflammatory stimulation by the viral infection, a rather immature host immune system) has no significant biological relevance during a real viral infection of carp. Secondly, it is possible that the role of ORF134 is strictly restricted to latency and viral reactivation. This hypothesis is inconsistent with the higher level of ORF134 expression observed during acute infection compared to those observed during latency and reactivation [26]. However, experiments are in progress to determine whether ORF134 deletion affects viral load during latency and/or the ability of the virus to reactivate and to be excreted. Thirdly, it may be that ORF134 expression product has a biological activity in zebrafish but not in common carp. This hypothesis is related to the still unknown origin of CyHV-3. Indeed, the rapid emergence of CyHV-3 in the common and koi carp population during the late 90s and the relatively low polymorphism existing between CyHV-3 isolates suggest that CyHV-3 is the consequence from a recent host-jump from a yet unidentified fish species to common and koi carp. According to this evolutionary scenario, it could be that ORF134 is functional in the CyHV-3 original host species and closely related species but not in the recently colonized common and koi carp species.

In conclusion, the present study addressed for the first time the in vivo role of a vIL-10 encoded by a member of the family *Alloherpesviridae*. It demonstrates that CyHV-3 ORF134 does not contribute significantly to viral growth in vitro or to virulence in vivo under the conditions tested. However, it is possible that this protein is important under circumstances that were not recapitulated in the present laboratory setting.

Abbreviations

AngHV-1: Anguilid herpesvirus 1; BAC: Bacterial artificial chromosome; CCB: *Cyprinus carpiocarpio* brain cell; CHX: Cycloheximide; CyHV-3: Cyprinid herpesvirus-3; Ct: Threshold cycle; DMEM: Dulbecco's modified essential medium; E: Early; EBV: Epstein-Barr virus; EGFP: Enhanced green fluorescent protein; *galK*: Galactokinase; HCMV: Human cytomegalovirus; IE: Immediate early; IFN: Interferon; IL-10: Interleukin-10; KHV: Koi herpesvirus; L: Late; MOI: Multiplicity of infection; MS: Mass spectrometry; ORF: Open reading frame; ORFV: Orf virus; PBS: Phosphate buffered saline; PAA: Phosphonoacetic acid; PFU: Plaque forming unit; Rhesus CMV: Rhesus cytomegalovirus; RT-PCR: Reverse transcription PCR; RT-qPCR: Real-time quantitative PCR; TK: Thymidine kinase; vIL-10: Virally encoded IL-10 homologues.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PO did most of the experiments. PO, KR, MB, AR, MR, GF, BC and AV contributed to the design of the study. PO and KR drafted the figures. BL and RW performed proteomic analyses. SC produced recombination cassettes. Statistical analyses were performed by KR. AV conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

PO is a research fellow of the Chinese Scholarship Council. This work was supported by a grant from the University of Liège and by grants of the "Fonds National Belge de la Recherche Scientifique" (FNRS) (R.FNRS.2165, -2697). KR and AV are members of the BELVIR consortium (IAP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).

Author details

[1]Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases (B43b), Faculty of Veterinary Medicine, University of Liège, Liège B–4000, Belgium. [2]Proteomic and Microbiology, CISMa, University of Mons, Place du parc, 20, Mons B-7000, Belgium. [3]Delphi Genetics SA, rue Antoine de Saint-Exupéry, 5, Gosselies B-6041, Belgium. [4]Femalon S.A, Rue du Travail, 16, Grâce-Hollogne B-4460, Belgium.

Received: 15 April 2013 Accepted: 10 June 2013
Published: 16 July 2013

References

1. Bretzinger A, Fischer-Scherl T, Oumouna M, Hoffmann R, Truyen U: **Mass mortalities in Koi carp, Cyprinus carpio, associated with gill and skin disease.** *Bull Eur Assoc Fish Pathol* 1999, **19**:182–185.
2. Hedrick R, Gilad O, Yun S, Spangenberg J, Marty G, Nordhausen R, Kebus M, Bercovier H, Eldar A: **A herpesvirus associated with mass mortality of**

juvenile and adult koi, a strain of common carp. *J Aquat Anim Health* 2000, **12**:44–57.
3. Hedrick RP: **Movement of pathogens with the international trade of live fish: problems and solutions.** *Rev Sci Tech* 1996, **15**:523–531.
4. Haenen O, Way K, Bergmann S, Ariel E: **The emergence of koi herpesvirus and its significance to European aquaculture.** *Bull Eur Assoc Fish Pathol* 2004, **24**:293–307.
5. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A: **Cyprinid herpesvirus 3.** *Emerg Infect Dis* 2010, **16**:1835–1843.
6. Aoki T, Hirono I, Kurokawa K, Fukuda H, Nahary R, Eldar A, Davison AJ, Waltzek TB, Bercovier H, Hedrick RP: **Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide.** *J Virol* 2007, **81**:5058–5065.
7. Davison AJ, Kurobe T, Gatherer D, Cunningham C, Korf I, Fukuda H, Hedrick RP, Waltzek TB: **Comparative genomics of carp herpesviruses.** *J Virol* 2013, **87**:2908–2922.
8. Waltzek TB, Kelley GO, Stone DM, Way K, Hanson L, Fukuda H, Hirono I, Aoki T, Davison AJ, Hedrick RP: **Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae.** *J Gen Virol* 2005, **86**:1659–1667.
9. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E: **The order Herpesvirales.** *Arch Virol* 2009, **154**:171–177.
10. McGeoch DJ, Rixon FJ, Davison AJ: **Topics in herpesvirus genomics and evolution.** *Virus Res* 2006, **117**:90–104.
11. Ilouze M, Dishon A, Kahan T, Kotler M: **Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses.** *FEBS Lett* 2006, **580**:4473–4478.
12. Savan R, Igawa D, Sakai M: **Cloning, characterization and expression analysis of interleukin-10 from the common carp, Cyprinus carpio L.** *Eur J Biochem* 2003, **270**:4647–4654.
13. Zhang DC, Shao YQ, Huang YQ, Jiang SG: **Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (Danio rerion).** *J Biochem Mol Biol* 2005, **38**:571–576.
14. Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, Geginat J: **Biology of interleukin-10.** *Cytokine Growth Factor Rev* 2010, **21**:331–344.
15. de Waal MR, Abrams J, Bennett B, Figdor CG, de Vries JE: **Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.** *J Exp Med* 1991, **174**:1209–1220.
16. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: **IL-10 inhibits cytokine production by activated macrophages.** *J Immunol* 1991, **147**:3815–3822.
17. Moore KW, de Waal MR, Coffman RL, O'Garra A: **Interleukin-10 and the interleukin-10 receptor.** *Annu Rev Immunol* 2001, **19**:683–765.
18. Couper KN, Blount DG, Riley EM: **IL-10: the master regulator of immunity to infection.** *J Immunol* 2008, **180**:5771–5777.
19. Slobedman B, Barry PA, Spencer JV, Avdic S, Abendroth A: **Virus-encoded homologs of cellular interleukin-10 and their control of host immune function.** *J Virol* 2009, **83**:9618–9629.
20. Kotenko SV, Pestka S: **Viral IL-10 variants**. In *Cytokine reference: a compendium of cytokines and other mediators of host defence.* Edited by Oppenheim JJ, Feldmann M. New York: Academic Press; 2001.
21. Hughes AL: **Origin and evolution of viral interleukin-10 and other DNA virus genes with vertebrate homologues.** *J Mol Evol* 2002, **54**:90–101.
22. van Beurden SJ, Forlenza M, Westphal AH, Wiegertjes GF, Haenen OL, Engelsma MY: **The alloherpesviral counterparts of interleukin 10 in European eel and common carp.** *Fish Shellfish Immunol* 2011, **31**:1211–1217.
23. Chang WL, Barry PA: **Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity.** *ProcNatlAcadSci USA* 2010, **107**:22647–22652.
24. Eberhardt MK, Chang WL, Logsdon NJ, Yue Y, Walter MR, Barry PA: **Host immune responses to a viral immune modulating protein: immunogenicity of viral interleukin-10 in rhesus cytomegalovirus-infected rhesus macaques.** *PLoS One* 2012, **7**:e37931.
25. Fleming SB, Anderson IE, Thomson J, Deane DL, McInnes CJ, McCaughan CA, Mercer AA, Haig DM: **Infection with recombinant orf viruses demonstrates that the viral interleukin-10 is a virulence factor.** *J Gen Virol* 2007, **88**:1922–1927.
26. Sunarto A, Liongue C, McColl KA, Adams MM, Bulach D, Crane MSJ, Schat KA, Slobedman B, Barnes AC, Ward AC, Walker PJ: **Koi herpesvirus encodes and expresses a functional interleukin-10.** *J Virol* 2012, **86**:11512–11520.
27. Costes B, Fournier G, Michel B, Delforge C, Raj VS, Dewals B, Gillet L, Drion P, Body A, Schynts F, Lieffrig F, Vanderplasschen A: **Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in Cyprinus carpio koi.** *J Virol* 2008, **82**:4955–4964.
28. Markine-Goriaynoff N, Gillet L, Karlsen OA, Haarr L, Minner F, Pastoret P-P, Fukuda M, Vanderplasschen A: **The core 2 β-1, 6-N-acetylglucosaminyltransferase-M encoded by bovine herpesvirus 4 is not essential for virus replication despite contributing to post-translational modifications of structural proteins.** *J Gen Virol* 2004, **85**:355–367.
29. Ilouze M, Dishon A, Kotler M: **Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes.** *Virus Res* 2012, **169**:98–106.
30. Ilouze M, Dishon A, Kotler M: **Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature.** *Virus Res* 2012, **169**:289–295.
31. Michel B, Leroy B, Stalin Raj V, Lieffrig F, Mast J, Wattiez R, Vanderplasschen AF, Costes B: **The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions.** *J Gen Virol* 2010, **91**:452–462.
32. Mastroleo F, Leroy B, Van Houdt RS, Heeren C, Mergeay M, Hendrickx L, Wattiez R: **Shotgun proteome analysis of Rhodospirillum rubrum S1H: integrating data from gel-free and gel-based peptides fractionation methods.** *J Proteome Res* 2009, **8**:2530–2541.
33. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M: **Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein.** *Mol Cell Proteomics* 2005, **4**:1265–1272.
34. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG: **Simple and highly efficient BAC recombineering using galK selection.** *Nucleic Acids Res* 2005, **33**:e36.
35. Costes B, Raj VS, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Bremont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin.** *J Virol* 2009, **83**:2819–2830.
36. Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP: **Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected Cyprinus carpio koi as assessed by real-time TaqMan PCR.** *Dis Aquat Organ* 2004, **60**:179–187.
37. Rakus KL, Irnazarow I, Adamek M, Palmeira L, Kawana Y, Hirono I, Kondo H, Matras M, Steinhagen D, Flasz B, Brogden G, Vanderplasschen A, Aoki T: **Gene expression analysis of common carp (Cyprinus carpio L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses.** *Dev Comp Immunol* 2012, **37**:65–76.
38. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method.** *Methods* 2001, **25**:402–408.
39. Dewals B, Boudry C, Gillet L, Markine-Goriaynoff N, De Leval L, Haig D, Vanderplasschen A: **Cloning of the genome of Alcelaphine herpesvirus 1 as an infectious and pathogenic bacterial artificial chromosome.** *J Gen Virol* 2006, **87**:509–517.
40. Fleming SB, McCaughan CA, Andrews AE, Nash AD, Mercer AA: **A homolog of interleukin-10 is encoded by the poxvirus orf virus.** *J Virol* 1997, **71**:4857–4861.
41. Lockridge KM, Zhou SS, Kravitz RH, Johnson JL, Sawai ET, Blewett EL, Barry PA: **Primate cytomegaloviruses encode and express an IL-10-like protein.** *Virology* 2000, **268**:272–280.
42. Drummer HE, Studdert MJ, Crabb BS: **Equine herpesvirus-4 glycoprotein G is secreted as a disulphide-linked homodimer and is present as two homodimeric species in the virion.** *J Gen Virol* 1998, **79**:1205–1213.
43. Costes B, Ruiz-Arguello MB, Bryant NA, Alcami A, Vanderplasschen A: **Both soluble and membrane-anchored forms of Felid herpesvirus 1 glycoprotein G function as a broad-spectrum chemokine-binding protein.** *J Gen Virol* 2005, **86**:3209–3214.
44. Rombout JH, Huttenhuis HB, Picchietti S, Scapigliati G: **Phylogeny and ontogeny of fish leucocytes.** *Fish Shellfish Immunol* 2005, **19**:441–455.
45. Chernoff AE, Granowitz EV, Shapiro L, Vannier E, Lonnemann G, Angel JB, Kennedy JS, Rabson AR, Wolff SM, Dinarello CA: **A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses.** *J Immunol* 1995, **154**:5492–5499.

doi:10.1186/1297-9716-44-53
Cite this article as: Ouyang *et al.*: The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. *Veterinary Research* 2013 **44**:53.

Experimental section

2nd chapter:

Development of a Safe and Efficacious Attenuated Recombinant Vaccine against Cyprinid Herpesvirus 3

In preparation

Ping Ouyang[1], Maxime Boutier[1], Maygane Ronsmans[1], Guillaume Fournier[1], Anca Reschner[1], François Lieffrig[2] and Alain Vanderplasschen[1*]

Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium[1]

CER Groupe, B-6900 Marloie, Belgium[2]

* Corresponding author. E-mail: a.vdplasschen@ulg.ac.be. Phone: +32 4 366 42 64

Preamble

Koi herpesvirus (KHV), also known as cyprinid herpesvirus 3 (CyHV-3), is the etiological agent of an emerging and mortal disease in common (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) carp. Since its emergence, in the late 1990s, this highly contagious and dreadful disease has caused severe economic losses in both common and koi carp culture industries worldwide [1].

The induction of herd immunity in fish farming is associated with key constraints. First, protective immunity should be induced early in life, *i.e.* as soon as the immune system is fully matured. The importance of inducing protective immunity as soon as possible in life is justified by the higher sensitivity of young fish to most diseases, by the aquaculture management (it is easier to vaccinate fish before they are distributed into nets or ponds) and by obvious economic reasons (in case of outbreak, the larger are the fish, the higher is the economic loss). Second, as a direct consequence of the former point, fish to be vaccinated are of a limited size (few centimetres) incompatible with handling and injection procedures. Finally, there is a strong constraint on the vaccine price that must be compatible with a positive "benefit – cost" balance.

Attenuated vaccines appear to be the most appropriate for mass vaccination of carp. Attenuated anti-CyHV-3 vaccine candidates have been produced by serial passages in cell culture of a pathogenic strain. A vaccine strain candidate was further attenuated by UV irradiation in order to increase random mutations throughout the genome [2]. Currently, an attenuated vaccine developed using this approach has been manufactured by KoVax Ltd. (Jerusalem, Israel) [3]. This vaccine was available temporarily for immersion vaccination of common and koi carp weighing 100 g or more in USA (Novartis) [4]. However, after just a year, Novartis decided to stop the sale of this vaccine. This vaccine has two major disadvantages: (*i*) the determinism of the attenuation is unknown; and consequently, reversions to a pathogenic phenotype cannot be excluded; (*ii*) the attenuated strain retains residual virulence that could be lethal for a portion of the vaccinated fish [5], particularly for small/young fish. Consequently, there is still a need for a safe and efficacious attenuated vaccine against CyHV-3.

The era of molecular biology is now allowing scientists to design idle attenuated replicating viral vaccines by targeting genes known to be involved in virulence and/or spreading and by causing to these genes defect that cannot be repaired by simple mutation. Recently, CyHV-3 genome has been cloned as an infectious bacterial artificial chromosome that can be mutated using prokaryotic recombination technologies [6]. While producing recombinant for ORF134, we unexpectedly obtained a clone deleted for ORF56 and ORF57 in addition to the expected ORF134 deletion. In comparison to its parental strain, this triple deleted recombinant (deleted for ORF56, 57 and 134) exhibited an attenuated phenotype and proved to be able to induce a protective immune response against a lethal challenge. Production of independent recombinants demonstrated that deletion of ORF56 and ORF57 were responsible for the safety/efficacy profile observed for the triple deleted recombinant.

Abstract

Cyprinid herpesvirus 3 is the causative agent of a lethal disease in common and koi carp. Since its emergence, it has caused severe economic losses worldwide creating the need for a vaccine. Taking advantage of the recent cloning of its genome as an infectious bacterial artificial chromosome (BAC), recombinant vaccine candidates were produced by deletion of a single gene. While producing such recombinant for open reading frame (ORF) 134, we unexpectedly obtained a clone additionally deleted for ORF56 and ORF57. Interestingly, this triple deleted recombinant exhibited an attenuated profile *in vivo*. To confirm that the triple deletion was indeed responsible for the phenotype observed and to determine the contribution of ORF134 deletion in the attenuation, a double ORF56-ORF57 deleted recombinant and an independent triple ORF56-ORF57-ORF134 recombinant were produced and tested *in vivo*. These experiments demonstrated that ORF56-ORF57 deletion was responsible for the attenuation and that ORF134 deletion did neither contribute significantly to the attenuation nor influence the adaptive immune response induced by the infection. To further investigate the adaptive immune response induced by the ORF56-ORF57 deleted strain, fish previously vaccinated with this strain were challenged with a wild type strain expressing luciferase as a reporter gene. These results suggested that the vaccine strain candidate induces a protective immunity able to prevent the entry of the challenging virus into vaccinated fish cells.

Key words: Cyprinid herpesvirus 3 (CyHV-3), attenuated vaccine, gene deletion.

Introduction

In the late 1990s, a highly contagious and virulent disease began to cause important economic losses in both common carp (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) industries worldwide [1]. Common carp is a freshwater fish widely cultivated for human consumption with a continuous increase of its world production over the last 30 years. The world production of common carp was estimated around 3.4 million metric tons in 2010 (Food and Agriculture Organization of the United Nations). The other susceptible host koi, are ornamental, colourful fish which are grown for personal pleasure and competitive exhibitions [7]. Initially called Koi herpesvirus (KHV) according to its morphological resemblance to viruses belonging to the order *Herpesvirales*, the agent was then known as carp interstitial nephritis and gill necrosis virus (CNGV) because of the associated lesions [8]. Finally, it was renamed CyHV-3 based on the homology of its genome with those of previously described cyprinid herpesviruses [9]. The rapid spread of the virus has been attributed to the international fish trade and koi shows that occur all around the world.

Soon after the identification of CyHV-3 as the causative agent of the disease, an original protocol was developed to induce a protective adaptive immune response in carp [8]. This approach exploited the fact that CyHV-3 induces fatal infections only when temperature is between 18°C and 28°C. According to this protocol, healthy fish are exposed to the virus by cohabitation with sick fish for 3-5 days at permissive temperature (22°C-23°C). Following this period, the fish are transferred to ponds for 25-30 days at non-permissive water temperature (≈30°C). Challenge performed on the surviving fish showed partial protection (mortality rate of 39% compared to 82% for the control group). Despite its ingenuity, this procedure has several disadvantages as (i) it uses a pathogenic strain leading to a severe risk of spreading the virus among cultivated and wild carp population during, but also after, the acute infection, as fish that are protected may become latently infected and exhibit reactivation at a later stage; (ii) it induces a loss of 40% of the fish during the procedure and protection is nevertheless not optimal as mentioned above; (iii) the increase of water temperature and fish losses are very costly for the farmers [8].

Vaccination is the most appropriate way to protect fish against CyHV-3. Various attempts of vaccination against CyHV-3 were made using both inactivated and attenuated vaccine candidates. One trial using oral immunization with a formalin-inactivated virus entrapped into a liposome compartment showed partial protection against a lethal challenge [10]. Attenuated vaccine candidates have also been developed by serial passages of the CyHV-3 Israeli strain in cell cultures [2,5,8]. After more than 20 passages, the attenuated strain was cloned and several clones were subsequently tested and submitted to UV irradiation to increase random mutations as well as decrease the risk of reversion to a pathogenic virus [2,5]. Currently, a live attenuated vaccine developed using this approach (Cavoy®) has been manufactured by KoVax Ltd. (Jerusalem, Israel) and is available for immersion vaccination of common and koi carp in USA (Norvartis Animal Health). This vaccine has three major

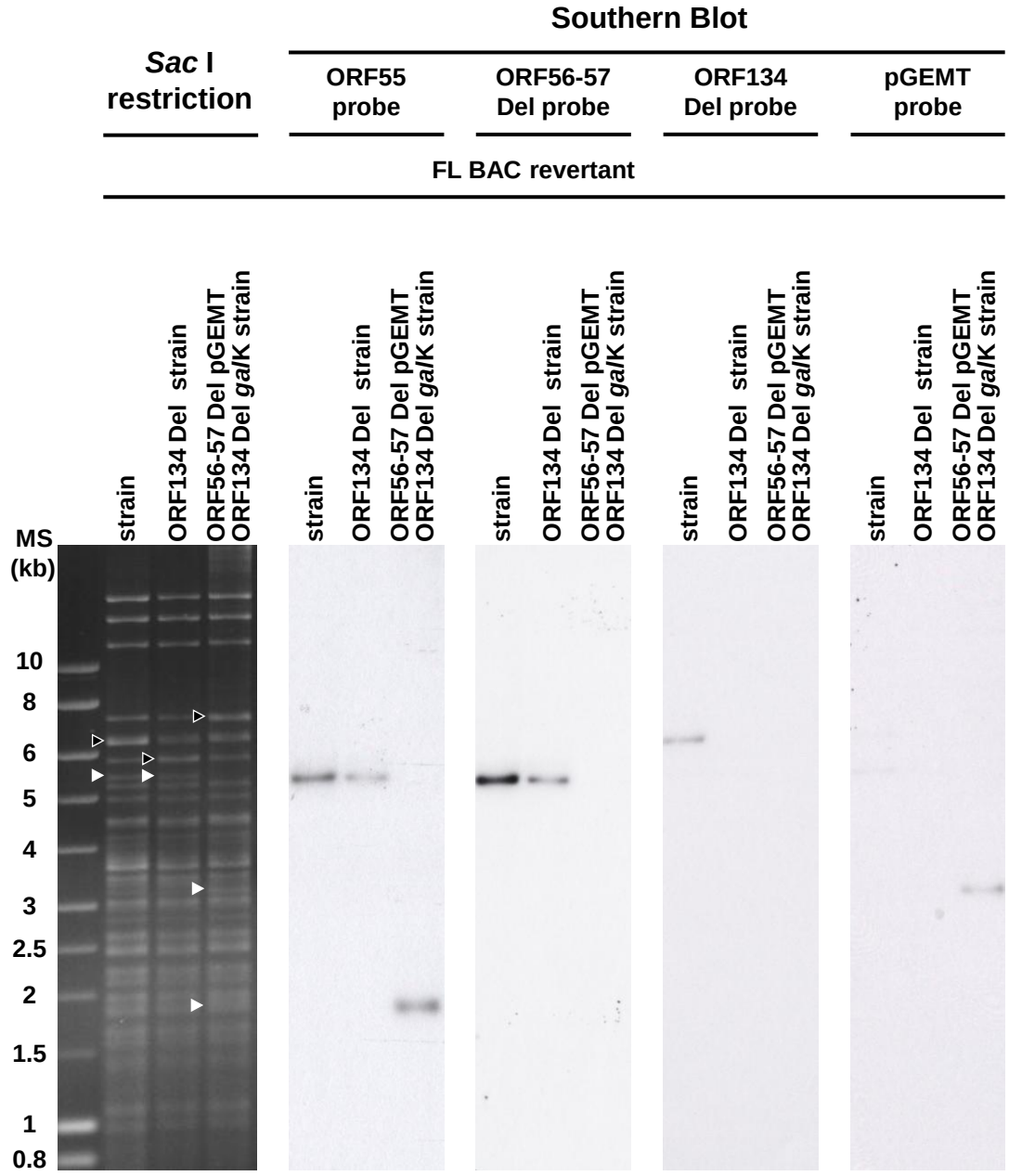


Figure 1. Molecular characterization of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain. The indicated strains were analyzed by *Sac* I restriction (left panel) and by Southern blot using ORF55, ORF56-57 Del, ORF134 Del and pGEMT probes. Black and white arrowheads indicate fragments containing the ORF134 locus and ORF56-57 loci, respectively. Marker size (MS) are indicated on the left.

disadvantages: (i) the determinism of the attenuation is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded; (ii) the attenuated strain retains residual virulence that could be lethal for a proportion of the vaccinated fish (20-30% of mortality rate during the vaccination in some experiments) [5], particularly for small/young fish, what probably explains why the vaccine is restricted to carps weighing more than 100 g (Norvartis Animal Health); (iii) the level of protection against a lethal challenge does not reach 100% [5].

Another way to produce attenuated vaccine candidates consists to delete specific ORFs which may be responsible for virulence. Some trials were made using such method. Fuchs and collaborators (2011) generated recombinants deleted for viral enzymes involved in the metabolism of nucleotide by recombination using *in vitro* enhanced green fluorescent protein (EGFP) positive/negative selection. Three ORFs were targeted ORF55 (thymidine kinase; [TK]), ORF123 (deoxyuridine triphosphatase; dUTPase) and ORF141 (RNR subunit 1). The derived recombinants were replicating *in vitro*, demonstrating that these ORFs are non-essential, although deletion of ORF141 lead to smaller lysis plaques as well as a slower viral growth. Results from *in vivo* infection using ORF55 and ORF123 deleted strains and their revertant strains showed inconsistent results and none deleted strain was simultaneously safe and efficacious [11]. In another study, Costes and collaborators (2008) produced a strain deleted for ORF16, encoding a potential GPCR and, by insertion and excision of the BAC cassette in ORF55, a TK truncated strain [6]. Both ORF55 truncated strain and ORF16 deleted strains showed partially attenuated phenotypes. Nevertheless, the level of attenuation was insufficient to propose these recombinants as vaccine candidates.

Recently, CyHV-3 genome has been cloned as an infectious bacterial artificial chromosome that can be mutated using prokaryotic recombination technologies [6]. While producing recombinant for ORF134 using this method, we unexpectedly obtained a clone deleted for ORF56 and ORF57 in addition to the expected ORF134 deletion. In comparison to its parental strain, this triple deleted recombinant (deleted for ORF56, 57 and 134) exhibited an attenuated phenotype and proved to be able to induce a protective immune response against a lethal challenge. Production of independent recombinants demonstrated that deletion of ORF56 and ORF57 were responsible for the safety/efficacy profile observed for the triple deleted recombinant.

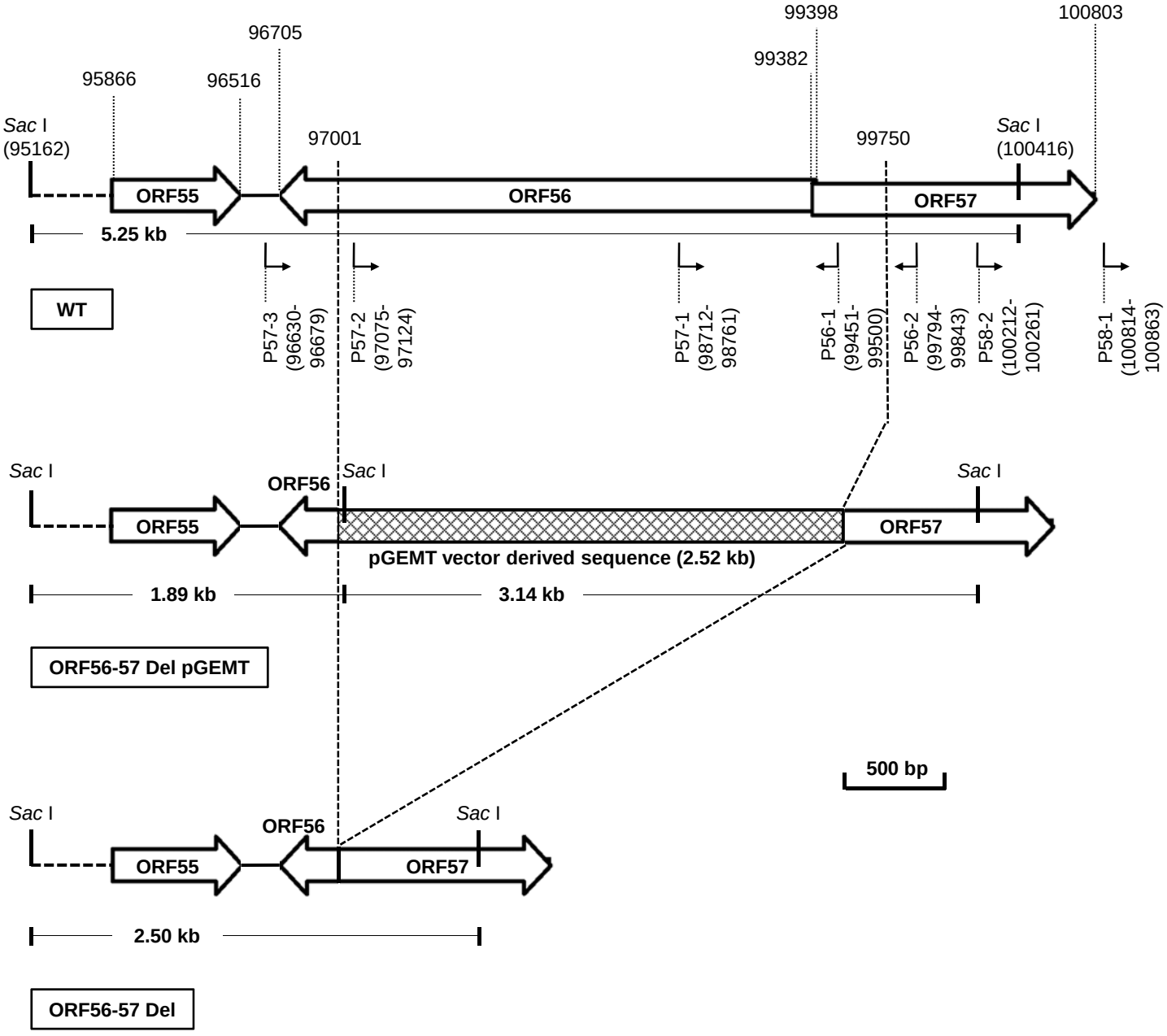


Figure 2. Schematic representation of the wild type ORF56-57 (WT), ORF56-57 Del pGEMT and ORF56-57 Del loci. ORFs are represented by white arrows. Predicted promoters for ORF56, ORF57 and ORF58 are represented by angular black arrows below the WT ORF56-57 genotype. Restriction *Sac* I sites and predicted restriction fragments (in kb) are described below each genotype. Coordinates are those of the CyHV-3 reference strain available in GenBank (Accession number DQ657948). The pGEMT vector derived sequence is represented by a hatched rectangle.

Results

Molecular characterization of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain

In the previous chapter, we produced a CyHV-3 recombinant strain that was deleted for ORF134 (encoding a viral IL-10). To reconstitute infectious viral particles, the FL BAC ORF134 Del *gal*K plasmid was co-transfected with pGEMT-TK as described in the materials and methods. This procedure led to an abnormal restriction profile for one of the clones produced (Figure 1). Indeed, the fragment of 5.25 kb encompassing ORF55 (in which the BAC plasmid was inserted) was absent of the restriction profile. Southern Blot analysis using ORF55 probe demonstrated that the ORF55 fragment was shorter than expected (1.9 kb). Moreover, by using pGEMT vector as probe, we demonstrated that a pGEMT vector derived sequence was inserted in the genome of this strain. The sequencing of this region of the genome confirmed the insertion of a pGEMT vector derived sequence (2.52 kb) but also demonstrated that a large part of CyHV-3 genome sequence was deleted (2.75 kb) (Figure 1). The region deleted encompassed most of ORF56 as well as the beginning of ORF57 (Figure 2). The southern blot performed with the ORF56-57 Del probe confirmed the correct deletion and demonstrated that the deleted sequence was not translocated elsewhere in the genome (Figure 1). The insertion of a *Sac* I restriction site originating from pGEMT vector explains the restriction profile observed in Figure 1. The deletion of ORF134 gene can be visualized directly on the restriction profile and was confirmed using an ORF134 Del probe. All together, the results presented above demonstrate that the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain is deleted for three ORFs: ORF56, ORF57 and ORF134.

Safety and efficacy profiles of FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain

The results presented above demonstrated that the FL BAC revertant ORF134 Del *gal*K strain is deleted for three ORFs: ORF56, 57 and ORF134 but is still able to replicate *in vitro*. Next, we investigated whether this combination of deletion could affect the virulence of this strain *in vivo*. Groups of 20 fish (average weight 7.19 g ± 3.40 g (mean ± SD), 6 months old) were infected by immersion for 2 h in water containing 4, 40, and 400 pfu/ml of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain or the FL BAC revertant strain used as control. A third group of fish was composed of mock-infected fish (Figure 3). Fish infected with the FL BAC revertant strain exhibited all clinical signs observed during CyHV-3 disease, beginning with folding of the dorsal fin, apathy and loss of appetite, hyperaemia, and later, skin and fin lesions. Independent of the dose tested, an important mortality was observed (with survival rate between 15 and 50%). In comparison to the wild type strain, the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain expressed an attenuated phenotype. Only few fish expressed mild clinical signs with the higher dose tested. These

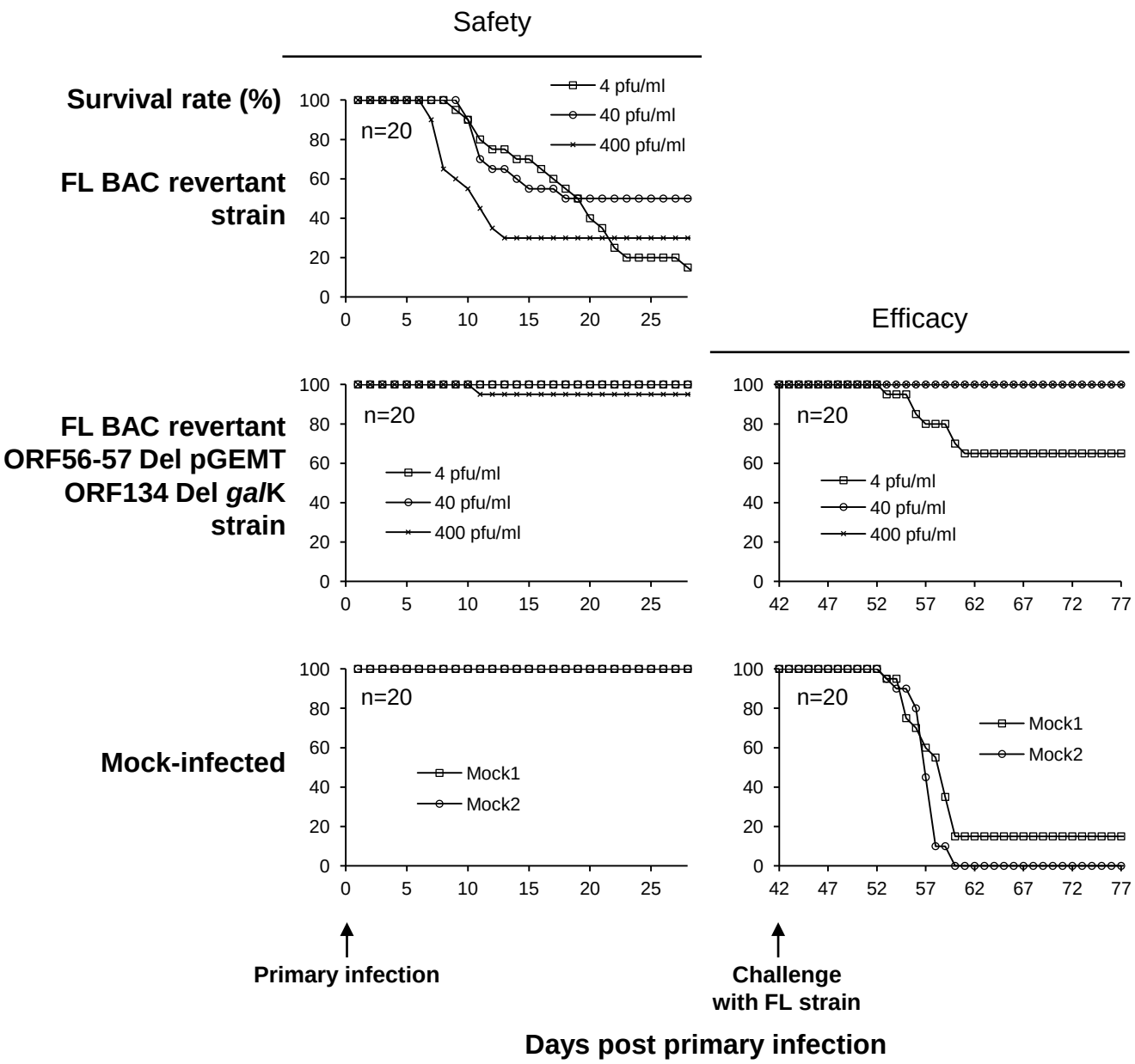


Figure 3. Safety and efficacy profiles of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain. The safety of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain was tested on common carp (n=20, average weight 7.19 ±3.4 g (mean ± SD), 6 months old). The FL BAC revertant strain and mock-infection were used as positive and negative controls, respectively. Fish were infected by immersion for 2h in water containing 4, 40, 400 pfu/ml of the indicated strains. Six weeks post-infection, fish that survived the primary infection and mock-infected fish were challenged by cohabitation with fish infected with FL strain. Percentages of surviving carp are expressed according to days post primary infection.

signs included folding of the dorsal fin and localised hyperaemia of the skin, but most fish expressing the infection remained active and fed normally. These observations were later confirmed by the survival rate which was very high in all groups (95-100%; one fish died in the 400 pfu/ml vaccinated group). More importantly, fish started to recover from the infection as soon as 8 dpi corresponding to the peak of mortality in the group infected with the wild type parental strain. Surviving fish were further challenged by cohabitation with fish infected with the FL strain. While mock-infected fish were very sensitive to this challenge (85-100% of the fish dying from the infection within 20 days post challenge), fish previously infected with the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain at the dose of 40-400 pfu/ml did not express the disease and exhibited 100% survival. Fish that were initially inoculated at the dose of 4 pfu/ml showed partial protection (65% of survival rate) (Figure 3).

Younger (small) fish have been shown to be more sensitive to CyHV-3 than older (large) fish. To further investigate the safety/efficacy phenotype of FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain, the experiment described in Figure 3 was reproduced with smaller fish (Figure 4). At the dose of 4 and 40 pfu/ml, neither clinical signs nor dead fish was observed. At the dose of 400 pfu/ml, few fish had minor and transient clinical signs (mainly consisting of skin hyperaemia) between days 5 and 8. However, one fish died at 9 days post vaccination. Fish infected with FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain, as well as mock-infected fish were divided in two groups of 15 fish. One group was challenged at 3 weeks post primary infection, while the second group was challenged at 6 weeks post primary infection. Challenges were performed by cohabitation with recently infected fish as described in the Materials and Methods. The results obtained after challenges at 3 and 6 weeks post primary infection led to comparable results (Figure 4). Infection performed at the dose of 4 pfu/ml did not induce a significant protection when compared to the mortality observed for mock-infected groups. At the dose of 40 pfu/ml, a partial protection (around 80% of survival rate) was observed while the dose of 400 pfu/ml induced full protection (100% survival) (Figure 4).

Production and characterization of CyHV-3 double and triple deleted recombinants

The FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain exhibits an attenuated phenotype. As demonstrated above, this strain is deleted for ORF56, ORF57 and ORF134. Based on previous study demonstrating that ORF134 is not essential for virulence *in vivo* (the first experimental chapter of the present thesis), these data suggested that the ORF56-57 deletion is responsible, on its own or in combination with ORF134 deletion, for the observed attenuated phenotype. However, as the full length genome sequencing of the strain was not performed, we cannot exclude the hypothesis that the phenotype observed is the consequence of additional undetected mutation(s). To exclude the latter hypothesis and to determine the respective roles of the ORF56-57

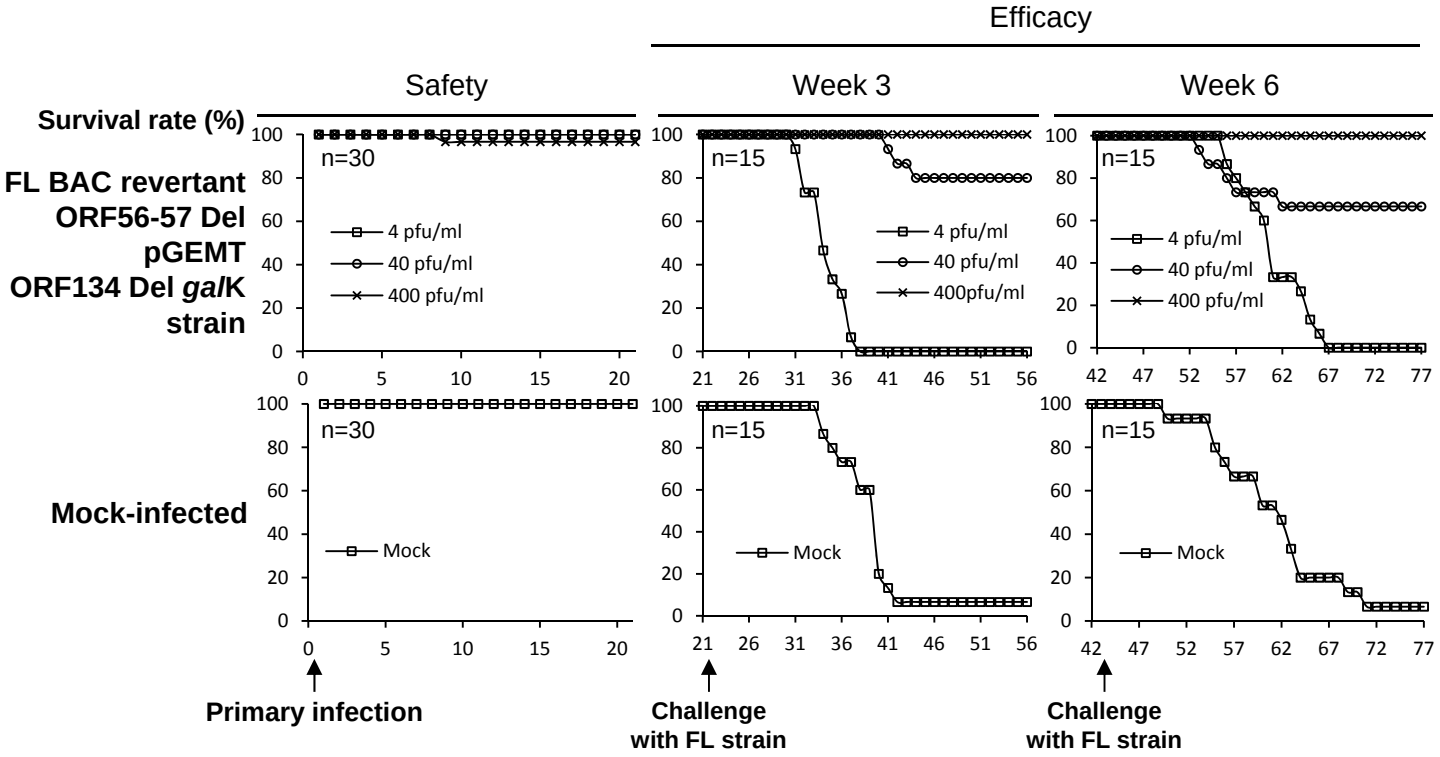


Figure 4. Safety and efficacy profiles of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain in small fish. The safety of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain was tested on common carp (n=30, average weight 1.35 ± 0.5 g (mean ± SD), 15 weeks old). Mock-infection was used as a negative control. Fish were infected by immersion for 2 h in water containing 4, 40, 400 pfu/ml. Surviving fish and mock-infected fish were challenged at 3 and 6 weeks post primary infection by cohabitation with fish infected with the FL strain. Percentages of surviving carp are expressed according to days post primary infection.

deletion and the ORF134 deletion in the attenuation observed, two independent recombinants were produced: one carrying the ORF56-57 deletion (double deleted recombinant) and one carrying the ORF56-57 deletion and the ORF134 deletion (triple deleted recombinant). These recombinants were produced using the strategy described in Figure 5. FL BAC revertant as well as FL BAC excised infectious forms were reconstituted from the FL BAC ORF56-57 Del plasmid and the FL BAC ORF56-57 Del ORF134 Del plasmid.

All strains were characterized by restriction fragment length polymorphism (RFLP) and southern blot analysis (Figure 6). Due to removal of the ORF56-57 Del sequence (2.75 kb), the ORF55 fragment was shorter (2.5 kb) as revealed by the ORF55 probe. Presence or absence of the BAC sequence (172 bp) allowed the discrimination between FL BAC excised and FL BAC revertant strains, respectively. Southern blot using ORF56-57 Del and ORF134 Del probes (corresponding to the deletions) demonstrated the absence of the deleted sequences in the recombinants (Figure 6). Finally, genome regions encompassing the mutated loci and the regions used to target recombinations were amplified by PCR and sequenced (data not shown). All analyses confirmed that the recombinants produced had the correct molecular structure.

Safety and efficacy profile of double and triple deleted recombinants

Groups of 30 fish were infected by immersion for 2 h in water containing 4, 40 and 400 pfu/ml of the FL BAC revertant ORF56-57 Del strain and FL BAC revertant ORF56-57 Del ORF134 Del strain (hereafter called double and triple deleted infected fish, respectively). A group of fish was infected with the FL BAC revertant strain used as a positive control for virulence (hereafter called wild type infected fish), while mock-infected fish were used as negative control (Figure 7A). For both the double and the triple deleted recombinants, the course of infection was similar to what we observed for the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain (Figure 3 and 4). Survival rate observed in wild type infected fish was between 40-47% depending of the dose used. In contrast, nearly all fish infected with the double and the triple deleted recombinants survived the infection. Indeed, only a single fish infected with the higher dose of the tripe deleted recombinant died as a consequence of the infection. These results demonstrated the key role of the ORF56-57 deletion in the safety profile observed for the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain.

Fish infected with the double and the triple deleted recombinants, as well as mock-infected fish were divided in two groups of 15 fish. One group was challenged at 3 weeks post primary infection, while the second group was challenged at 6 weeks post primary infection. Challenges were performed by cohabitation with recently infected fish as described in the Materials and Methods. Results obtained after challenges at 3 and 6 weeks post primary infection led to comparable results. Nearly all fish vaccinated at the dose of 40 and 400 pfu/ml were protected against this lethal challenge (only 2 dead fish out of 119 fish challenged) whereas only 2 mock-infected fish survived out of 60

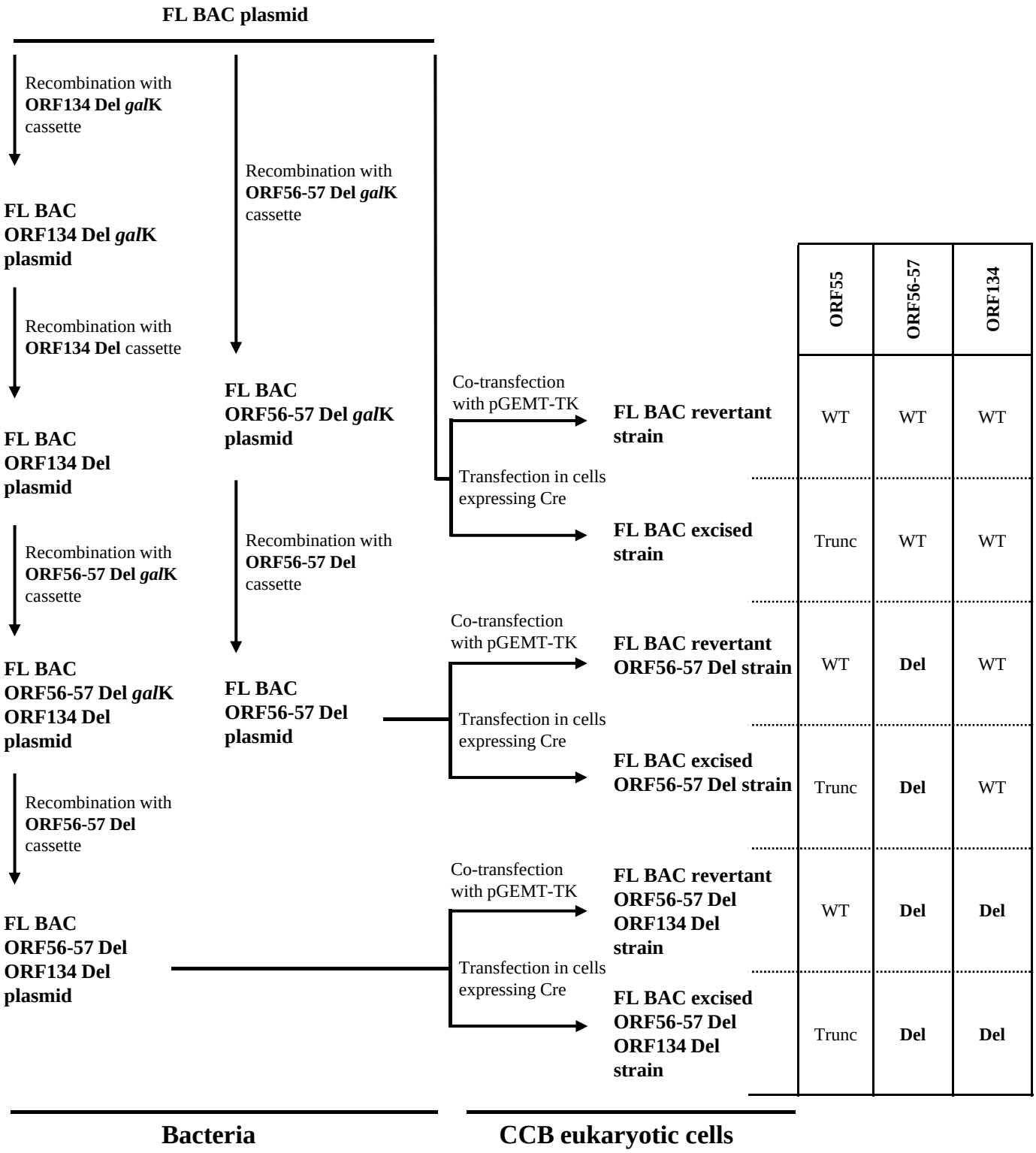


Figure 5. Flowchart of the stages for the production of double and triple deleted recombinants. The region of ORF56-57, as identified in Figure 1, was replaced by a *gal*K gene using homologous recombination with ORF56-57 Del *gal*K cassette in both FL BAC plasmid and FL BAC ORF134 Del plasmid. The *gal*K gene was then removed by homologous recombination with a synthetic DNA sequence corresponding to CyHV-3 genome regions flanking the ORF56-57 deletion (ORF56-57 Del cassette). To reconstitute infectious virus with a wild type TK locus (FL BAC revertant strains), the recombinant plasmid was co-transfected in CCB cells with the pGEMT-TK plasmid. To reconstitute infectious virus with a truncated form of TK (FL BAC excised strains), the recombinant plasmid was transfected in CCB cells expressing Cre recombinase. The right part of the figure summarizes the genotype of the various strains produced.

challenged fish. Protection observed for fish infected at the dose of 4 pfu/ml was partial as observed in previous experiments. All these experiments performed with the FL BAC revertant forms of the double and triple deleted recombinants were reproduced with the FL BAC excised forms. These experiments confirmed the results obtained (Figure 7B).

The results of the experiments presented above demonstrated that the ORF56-57 deletion explains the safety/efficacy phenotype observed for the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain. They also demonstrated that the additional deletion of ORF134 and/or truncation of ORF55 do not improve the safety/efficacy profile.

Characterization of the immune protection conferred by the FL BAC revertant ORF56-57 Del strain using *in vivo* imaging system

Most vaccines confer an immune protection able to reduce, but not to block completely, viral replication in the vaccinated subject. Consequently, most vaccines confer a clinical protection but do not confer an absolute resistance (sterile immunity). In this last section, we investigated by using *in vivo* imaging system (IVIS), the ability of the immune response induced by the FL BAC revertant ORF56-57 Del strain to inhibit the infection by a challenger wild type virus expressing luciferase under control of the HCMV IE promoter (Figure 8). Fish were infected by immersion for 2 h in water containing 40 and 400 pfu/ml of the FL BAC revertant ORF56-57 Del strain. Mock-infected fish were used as control. The course of infection was similar to what we observed before. Six weeks after the primary infection, fish were challenged by immersion in water containing the FL BAC revertant 136 LUC. At different times post challenge, fish were analyzed by IVIS for detection of bioluminescence revealing infection (sensitivity and/or permissiveness of host cells). As early as two days post infection, most naïve fish (5 out of 6) that were challenged with the FL BAC revertant 136 LUC were detected as positive for bioluminescence revealing viral infection. On day 4 and 8 post-challenge, all fish tested were positive and expressed a signal increasing with time post-challenge. In contrast, fish immunized against the FL BAC revertant ORF56-57 Del strain prior to the challenge expressed no significant level of bioluminescence with exception of one fish on day 2 (immunized at the dose of 40 pfu/ml). All together, these data suggest that the FL BAC revertant ORF56-57 Del strain has some potential as a vaccine strain candidate.

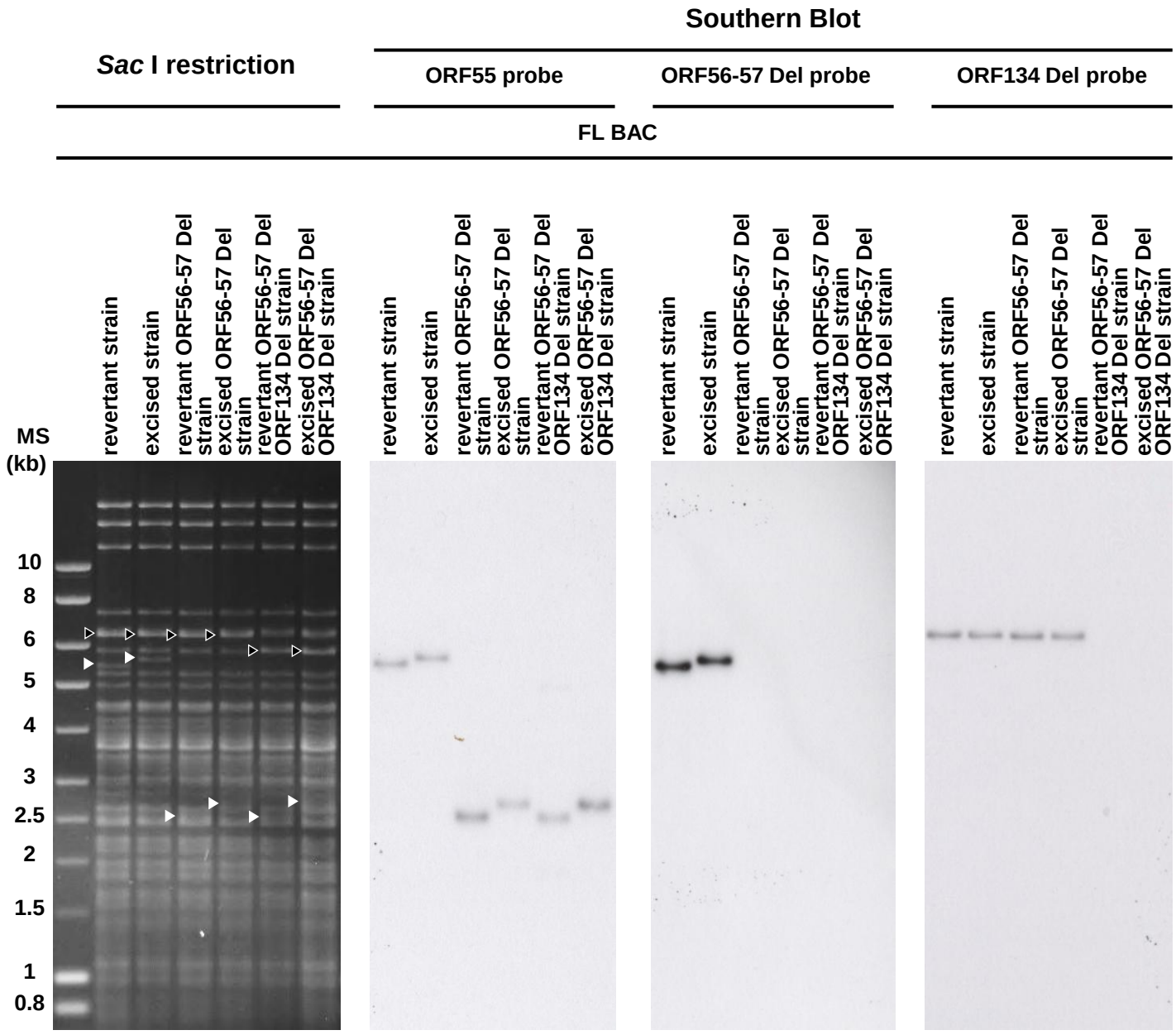


Figure 6. Molecular characterization of double and triple deleted recombinants. The indicated strains were analyzed by *Sac* I restriction (first panel) and by Southern blot using ORF55, ORF56-57 Del and ORF134 Del probes. Black and white arrowheads indicate fragments containing ORF134 locus and ORF56-57 loci respectively. Marker size (MS) are indicated on the left.

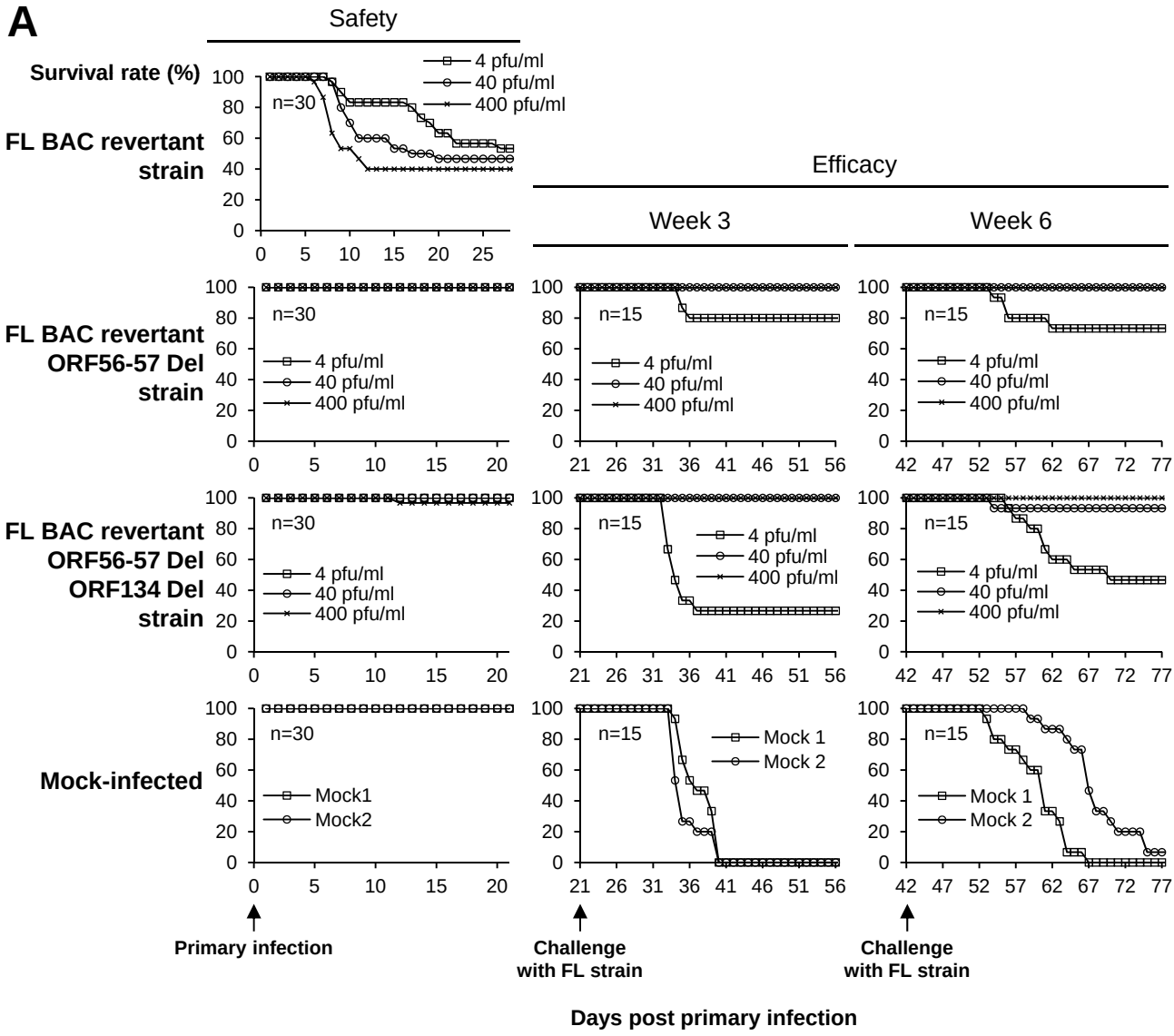


Figure 7. Safety and efficacy of double and triple deleted recombinants. Panels A and B present the testing of TK revertant and TK truncated recombinants, respectively. The safety of the indicated recombinants were tested on common carp (n=30, average weight 3.77 ± 1.97 g (mean ± SD), 7 months old). The FL BAC revertant strain and mock-infection were used as positive and negative controls, respectively. Fish were infected by immersion for 2 h in water containing 4, 40, 400 pfu/ml. Surviving fish and mock-infected fish were challenged at 3 and 6 weeks post primary infection by cohabitation with fish infected with the FL strain. Percentages of surviving carp are expressed according to days post primary infection.

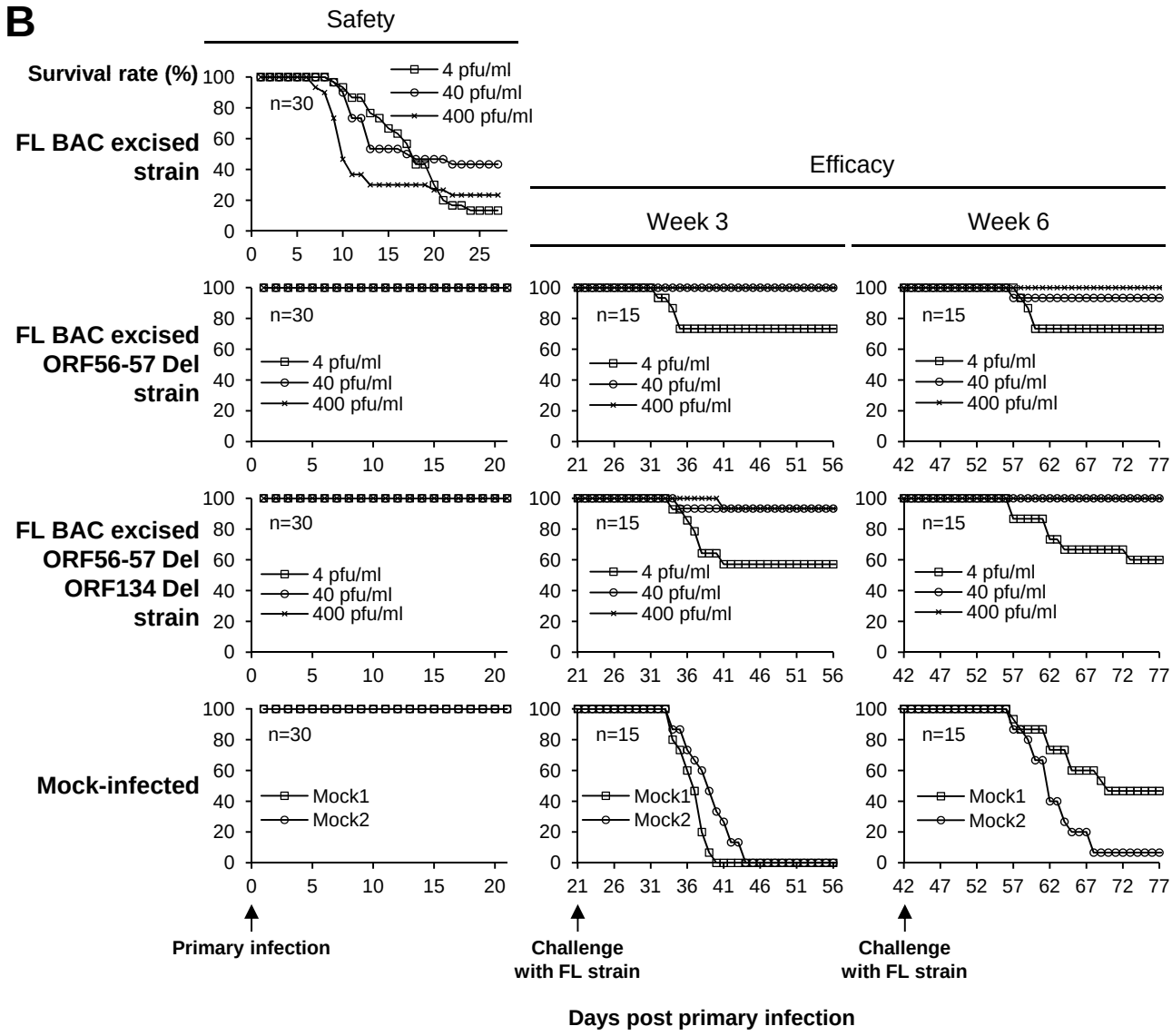


Figure 7. Safety and efficacy of double and triple deleted recombinants. Panel B see legend under Figure 7 panel A.

Discussion

CyHV-3 is a highly pathogenic virus causing devastating losses in cultured and wild common carp populations, and in ornamental koi carp. Due to the economic losses caused to aquaculture and the rapid spread of the virus worldwide, researches in the field were rapidly oriented to the development of diagnostic and prophylactic tools [1]. Vaccination is likely to be the most efficacious way to reduce spreading of viral infections and to protect fish from its deleterious effects. Attenuated vaccines seem to be particularly adapted for vaccination of carp against CyHV-3. Firstly, attenuated vaccines stimulate both humoral and cellular immune responses, the latter is known to be of particular importance for immune protection against viral diseases. Secondly, an attenuated vaccine is usually easy to produce, requires lower vaccine dose to induce immune protection and is frequently active after a single dose. These features make them well adapted for mass vaccination [12]. Thirdly, immunity raised by an attenuated vaccine is usually long-lasting [5]. A CyHV-3 attenuated vaccine was developed using attenuation through serial passages *in vitro* and UV irradiation to induce random mutations [2,5,8]. The determinism of its attenuation is unknown and consequently the risk of reversion cannot be evaluated or excluded. The present study was devoted to the rational development of a recombinant attenuated vaccine against CyHV-3.

The publication of the CyHV-3 complete genome [13] (recently updated and compared to sequences of other members of the genus *Cyprinivirus* ; family *Alloherpesviridae* ; order *Herpesvirales* [14]), combined with its recent cloning as a BAC, allowed the production of recombinants deleted for targeted genes [6]. During the production of recombinant strain deleted for ORF134 encoding an IL-10 homologue [15], an additional double deletion (ORF56-57) occurred during the process of removal of the BAC cassette. Interestingly, this triple deleted recombinant (deleted for ORF56, ORF57 and ORF134 and possibly undetected mutations) replicated efficiently *in vitro*, exhibited an attenuated profile *in vivo* and induced an immune protection against a lethal challenge (Figure 3). This observation was the starting point of the present study. Based on a rational approach, various recombinant strains were produced to unravel the determinism of the attenuation of this strain.

As the single deletion of ORF134 was shown to have no effect on the virulence of CyHV-3 *in vivo* [15], the double deletion (ORF56-57) was supposed to be responsible for the severe attenuation observed for the FL BAC ORF56-57 Del pGEMT ORF134 Del *gal*K. Nevertheless, the deletion of ORF134 encoding an IL-10 homologue could contribute to the observed attenuated phenotype. Moreover, we could not exclude the unlikely hypothesis that the triple deleted recombinant encoded undetected additional mutations that could have been responsible for the attenuated phenotype. To confirm that the double ORF56-57 deletion was indeed responsible for the phenotype observed and to determine the contribution of ORF134 deletion in the attenuation, a double ORF56-57 deleted recombinant and an independent triple ORF56-57/ORF134 deleted recombinant were produced and

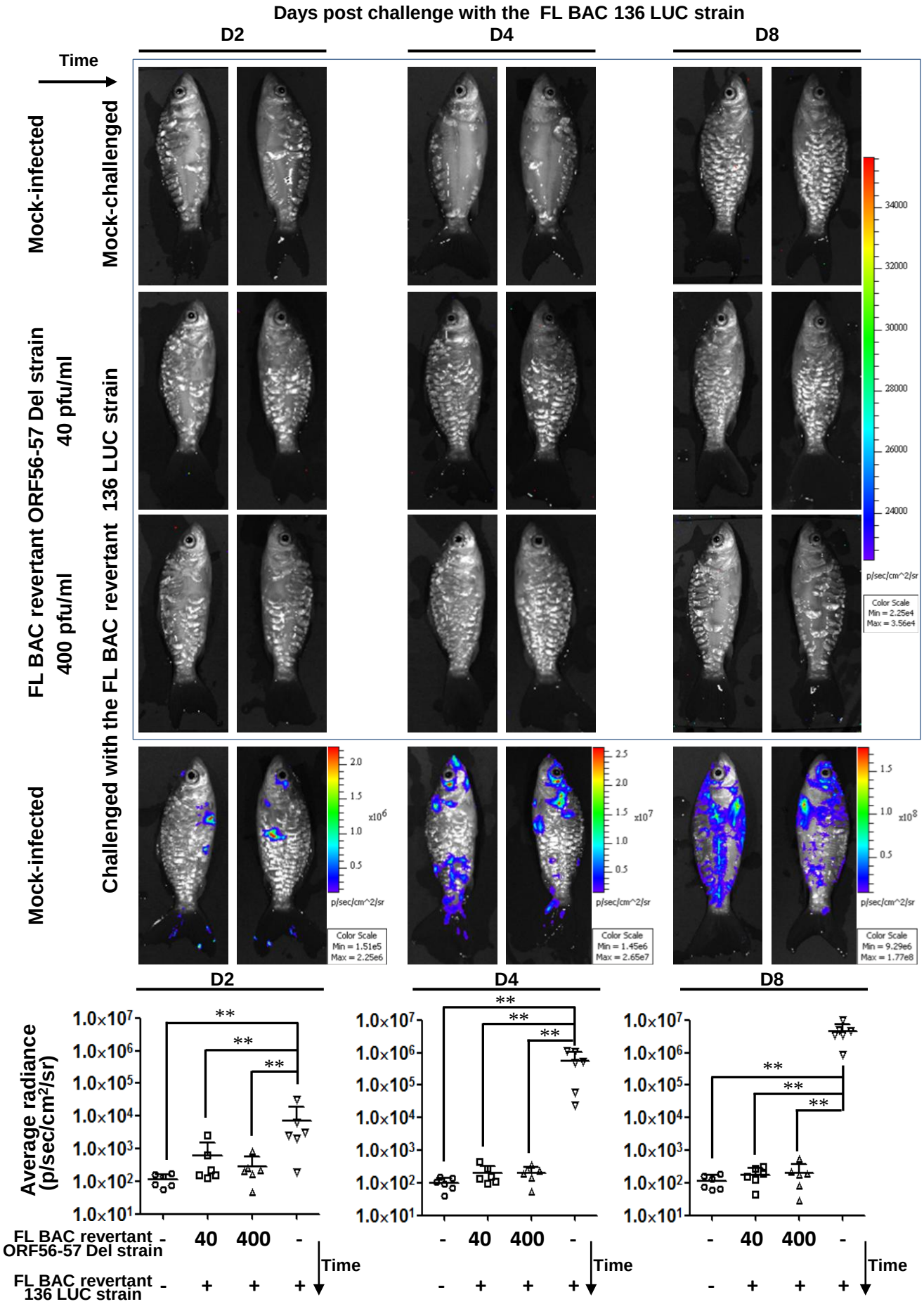


Figure 8. Immune protection conferred by the FL BAC revertant ORF56-57 Del strain as revealed by IVIS. Common carp (n=20, average weight 13.82 ± 5.00 g (mean ± SD), 9 months old) were infected by immersion for 2 h in water containing 40 or 400 pfu/ml of the FL BAC revertant ORF56-57 Del strain or were mock-infected. None of the fish died from this primary infection. At 6 weeks post primary infection, fish were challenged by immersion for 2 h in water containing 200 pfu/ml of the FL BAC revertant 136 LUC strain. At the indicated time post challenge, fish (n=6) were analysed by IVIS. The upper part of the figure presents representative pictures obtained for each group and time point. The lower part presents the average radiance (+ SD) measured on the entire fish body surface per group and time point, as well as the individual measures obtained. Differences with p-value lower than 1 % are marked (**).

tested *in vivo* (Figures 5 to 7). These experiments revealed that ORF134 deletion did neither contribute significantly to the attenuation observed nor influence the adaptive immune response induced by the infection. Importantly, these experiments demonstrated that ORF56-57 deletion was responsible for the attenuation observed. Moreover, the comparison of the FL BAC excised and revertant forms (respectively encoding a truncated or revertant ORF55 locus) of the double and triple deleted recombinants also revealed that truncation of ORF55 did not improve the safety and/or efficacy of the strains (Figure 7, compare sections A and B). Notably, the partial attenuation due to truncation of ORF55 in a wild type FL background observed previously could not be reproduced in these experiments. This discrepancy is likely due to the fact that, in contrast to the quoted study, we used fish in exponential growth phase and thereby containing a large proportion of dividing cells expressing high level of cellular TK. Other differences in experimental procedures (we used common carps instead of koi carps, infection by immersion instead of infection by IP injection) [6] could also explain the differences observed.

Taking into account all the recombinants deleted for ORF56-57 produced, about 420 fish with an average weight lower than 10 g were infected with 3 different doses (4, 40 and 400 pfu/ml by immersion). Out of these 420 fish, only two fish died after infection demonstrating the safety of the vaccine candidate (all mortalities reported). Concerning the efficacy, fish vaccinated at 40-400 pfu/ml and challenged with the FL strain showed very high level of protection, only 4 fish died out of 280 fish challenged. Experiment performed on very small fish further demonstrated the safety conferred by deletion of the ORF56-57 loci (Figure 4). Compared to the CyHV-3 attenuated vaccine available in USA (produced by KoVax Ltd, Israel), these results are very promising. Indeed, safety and efficacy of this vaccine are much lower than the vaccine candidate developed in the present study. Firstly, this vaccine has been tested at doses of 100-200 pfu/fish by IP and 10-40 pfu/ml by immersion and already raised safety concerns at these relatively low doses (the vaccine can be lethal for up to 30% of vaccinated fish weighting less than 50 g). This probably explains why this vaccine was initially registrated for fish weighing more than 100 g (Norvartis Animal Health). However, very recently after just a year on the market, Novartis decided to stop the distribution of this vaccine officially for marketing reasons. In addition to safety issues, this vaccine exhibited efficacy performance below those observed in the present work; indeed, the KoVax vaccine was never reported to confer full protection [2,5,8].

The candidate vaccine developed in the present study presents the major advantage to be the product of a rational genetic deletion rather than the result of random mutations. The consequences to precisely know the genetic origin of attenuation can be seen at several levels. The development of an attenuated vaccine in aquaculture is restricted by major safety concerns as these vaccines are usually released directly into the water [12]. Knowledge of the gene deleted and the type of mutation performed to invalidate the gene is crucial to estimate the risk of reversion to a pathogenic genotype. The vaccine candidate developed in this work fulfills this requirement. While point mutations acquired

by serial passages in cell cultures are likely to be reversed, reversion of the deletion performed in our vaccine candidate is impossible.

The precise knowledge of the genetic deletions present in our vaccine candidate allows the parallel development of diagnostic tools allowing the differentiation between infected and vaccinated animals based on genetic, antigenic and/or serologic assays. Another advantage of the vaccine candidate developed in this work, is that it has the potential to be used as vector for expression of key protective antigens of other pathogens. The expression cassette encoding these antigens will have to be inserted in place of the ORF56-57 deletion performed. As CyHV-3 has been shown to infect carp but also other species of cyprinid fish, it could in theory be used as an expression vector in all sensitive species. For example, spring viraemia of carp (SVC) is caused by a rhabdovirus responsible for mortalities in cyprinid species and against which no vaccine is available. As vaccination using glycoprotein G in delivery system such as DNA or vector based vaccine are frequent and efficacious in this viral family [16,17], the SVC could be a potential future candidate for vaccine development using CyHV-3 as a vector.

Orthologues of ORF57 have been reported in three viral species infecting fish (CyHV-1 and 2, AngHV-1) [14], two of them are responsible for severe diseases in their respective hosts. Indeed, CyHV-2, also known as goldfish haematopoietic necrosis virus, is responsible for a severe disease initially reported in goldfish (*Carassius auratus auratus*) [18], but recently emerging in gibel carp (*Carassius auratus gibelio*) [19]. In addition, AngHV-1 that infects European and Japanese eel (*Anguilla anguilla* and *Anguilla japonica*) is responsible for mortalities up to 30% in cultured and wild eel populations [20]. An additional homologue of ORF57 has also been described in a more distant viral species: the crocodilepox virus, an unclassified member of the subfamily *Chordopoxvirinae* of the family *Poxviridae* [21]; what suggests an ancestral origin of the gene. Consequently, putative other homologues of ORF57 are likely to be reported in the future. The basis of the present work identifies ORF57 as a first choice gene candidate to be deleted in order to produce attenuated vaccine for viruses encoding an ORF57 homologue.

In conclusion, the present work established the basis for further investigations on the use of ORF56-57 deleted CyHV-3 recombinants as attenuated vaccine against CyHV-3. The perspectives of the present study are related to both applied and fundamental sciences; they can be divided into four main topics: (i) to further investigate which ORF contribute to the attenuation observed: ORF56 and/ or ORF57? (ii) to further investigate the potential of the FL BAC revertant ORF56-57 Del strain as an anti-CyHV-3 vaccine candidate; (iii) to investigate the potential of this vaccine candidate as an expression vector for vaccination against heterologous pathogenic agents; and (v) as a subject of fundamental research to study the roles of ORF56 and/ or ORF57 in CyHV-3 infection.

Table 1. Primers and probes

Probe/cassette name	Primer name	Sequence (5'- 3')	Coordinates according to DQ657948
Primers for Southern blot analysis			
CyHV-3 ORF55	ORF55InF	AGCGCTACACCGAAGAGTCC	95990-96009
	ORF55stopR	TCACAGGATAGATATGTTACAAG	96516-96494
CyHV-3 ORF 56-57 Del probe	ORF56-57Pr5F	GGTACAAGACGGCCTGCTG	97247-97265
	ORF56-57Pr9R	GCCAGCACGTAGAGCTTGTG	99686-99667
CyHV-3 ORF134 Del probe	ORF134InF	GGTTTCTCTTTGTAGTTTTCCG	229362-229383
	ORF134InR	CACCCCAACTTTTGAGACAAC	229795-229765
*gal*K probe	*gal*KF2	AGGTGAGGAACTAAACCCAG	
	*gal*KR2	GATAAAGCTGCTGCAATACG	
Primers for amplification of recombination cassettes			
ORF 134 Del *gal*K cassette	ORF134 *gal*K F	ATGTTCCTTGCAGTGCTACTAACCGCG ACCATCTTCTTCGAGGCTCGGGG *CCTGTTGACAATTAATCATCGGCA*	229791-229840
	ORF134 *gal*K R	TCAATGTTTGCGCTTGGTTTTCATGTTC TTGACGTCTTTTGCGACCAGGA *TCAGCACTGTCCTGCTCCTT*	229217-229266
ORF 56-57 Del *gal*K cassette	ORF56-57 *gal*K F	GTCCCTCGACAGCCCCAGCCCGCACA GCAGTCGCCACTCTTCCCTGTTGA *TCAGCACTGTCCTGCTCCTT*	96951-97000
	ORF56-57 *gal*K R	AACCCGTACACGACGCGCTCAAGCAG CTTGATCTTGACGACGTCGTGCAC *CCTGTTGACAATTAATCATCGGCA*	99800-99751

Underlined: 50bp corresponding to CyHV-3 sequence
Italic: sequence corresponding to *gal*K gene

Materials and Methods

Cells and viruses

Cyprinus carpio brain (CCB) cells [22] were cultured in minimum essential medium (Invitrogen) containing 4.5 g/liter glucose (D-glucose monohydrate; Merck) and 10% fetal calf serum. The cells were cultured at 25°C in a humid atmosphere containing 5% CO_2. The CyHV-3 FL strain was isolated from the kidney of a fish that died from CyHV-3 infection (CER, Marloie, Belgium) and was previously used to produce the FL BAC plasmid [6]. The FL BAC revertant 136 LUC virus expressing luciferase as a reporter gene has been described earlier [23].

Fish

Common carp (*Cyprinus carpio*) (CEFRA, University of Liège, Belgium), were kept in 60 L tanks at 24°C. Water parameters were checked weekly. Microbiological, parasitical and clinical examinations of the fish just before the experiments demonstrated that they were fully healthy.

Inoculation of fish

After a period of acclimation of 2 weeks, primary infection was done by immersion of fish in 1-2 liters (depending on fish size) of water containing the virus (infectious doses of 4, 40 or 400 plaque forming unit [pfu]/ml) for 2 h. At the end of the incubation period, the fish were returned to 60 L tanks and then observed daily for clinical signs and mortality. Challenge of survival fish was performed at 21 days and/or 42 days post primary infection by cohabitation with infected fish. Two fish, freshly infected by immersion for 2 h in water containing 200 pfu/ml of the FL strain, were released in each tank to be challenged. The animal study was accredited by the local ethics committee of the University of Liège, Belgium (Laboratory accreditation N°1610008, protocol N°1059).

Production of double and triple deleted recombinants

Recombinants were produced using BAC cloning and prokaryotic recombination technologies. The different recombinant plasmids were produced using a two-step galactokinase *(gal*K) positive/negative selection in bacteria as described previously [24]. The primers used are described in Table 1. Production of double and triple deleted recombinants encoding the accidental deletion of ORF56-57 observed in the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del *gal*K strain (Figure 2) was done by using FL BAC plasmid and FL BAC ORF134 Del plasmid as parental plasmids, respectively; and following the strategy described in Figure 5 [15]. The first recombination process consisted to replace ORF56-57 by a *gal*K gene (positive selection) resulting in the FL BAC ORF56-57 Del *gal*K and FL BAC ORF56-57 Del *gal*K ORF134 Del plasmids. Recombination was performed using the ORF56-57 Del *gal*K recombination cassette consisting of the *gal*K gene flanked

by 50 base pair (bp) sequences homologous to CyHV-3 genome regions flanking ORF56-57 deletion. The ORF56-57 Del *gal*K recombination cassette was produced by PCR (primers 56-57 *gal*K F and 56-57 *gal*K R) using the p*gal*K vector as template. Primer 56-57 *gal*K F consisted of nucleotides 96951-97000 (50 bp) of CyHV-3 genome and nucleotides 1212-1231 (20 bp) of the pgalK vector. Primer 56-57 *gal*K R consisted of nucleotides 99751-99800 (50 bp) of the CyHV-3 genome and nucleotides 1-24 (24 bp) of the p*gal*K vector. The 50 bp sequences of the ORF56-57Del *gal*K corresponding to CyHV-3 genome were used to target homologous recombination in bacteria. The second recombination process (*gal*K negative selection) consisted to remove the *gal*K gene through recombination with the ORF56-57 Del cassette leading to the production of the FL BAC ORF56-57 Del and the FL BAC ORF56-57 Del ORF134 Del plasmids. The ORF56-57 Del cassette consisted of 250 bp upstream (coordinates 96751-97000) and 249 bp downstream (99751-100000 with deletion of base 99760) of ORF56-57 deletion. To reconstitute infectious virus, the BAC plasmids (FL BAC, FL BAC ORF56-57 Del, FL BAC ORF56-57 Del ORF134 Del) were co-transfected into CCB cells using Polyethylenimine (ratio 3 µg of Polyethylenimine for 1 µg of DNA) either with the pGEMT-TK plasmid or with pEFIN3 NLS Cre (Molecular ratio 1:75) [6]. Transfection with pGEMT-TK plasmid induced recombination upstream and downstream the BAC cassette leading to its complete removal and consequently reversion to a wild type TK locus (FL BAC revertant strains). Transfection with pEFIN3 NLS Cre induced expression of a nuclear Cre recombinase and cre-loxP-mediated excision of the BAC cassette. Viruses reconstituted (FL BAC excised strains) by this procedure express a truncated TK locus due to the BAC sequence left (172 bp) in this locus. Plaques negative for EGFP expression (the BAC cassette encodes an EGFP expression cassette) were picked and amplified.

Genetic characterization of recombinants

RFLP were performed using *Sac* I digestion on 2.5µg of viral DNA. Digested DNA samples were then submitted to migration for18 hours (50 V – 500 mA). Southern blot analyses were then performed as described elsewhere (Markine-Goriaynoff *et al.*, 2004) using several probes. Viral DNA was also submitted to PCR analysis and sequencing of targeted regions.

Bioluminescence imaging

Imaging of firefly (Photinus pyralis) LUC was performed using an "*in vivo* imaging system" (IVIS) (IVIS®spectrum, Xenogen, USA) as described previously [23,25]. For *in vivo* analysis, fish were anesthetized with benzocaine (50 mg/L of water). Ten minutes before bioluminescence analysis, D-luciferin (150 mg/kg body weight) (Xenogen, USA) was administrated by intraperitoneal injection. Each fish was analyzed lying on its left and right side. All the images presented in this study were acquired using a field view of 15 cm, a 1 min exposure time, a binning factor of 4 and a f/stop of 1. Relative intensities of transmitted light from bioluminescence were represented as a pseudocolor

image ranging from violet (least intense) to red (most intense). Corresponding grey-scale photographs and color luciferase images were superimposed using the Living Image analysis software (Xenogen, USA). For quantitative comparisons, the Living Image software (Caliper Life Sciences) was used to obtain the total flux ($p.s^{-1}$) over each region of interest.

Statistical analyses

The differences in mortality induced by the CyHV-3 strains tested were analyzed using Kaplan and Meier survival analysis. Log-transformed bioluminescence data were analyzed using two-ways analysis of variance. Fish groups, days after challenge and the interaction between these 2 factors were tested. Results are presented as least-square means and standard errors, and p-values lower than 0.05 and 0.01 were reported as significant (*) and very significant (**), respectively.

Acknowledgments

PO is a research fellow of the Chinese Scholarship Council. This work was supported by a grant from the University of Liège and by grants of the 'Fonds National Belge de la Recherche Scientifique' (FNRS) (R.FNRS.2165, -2697). AV is a member of the BELVIR consortium (IAP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).

References

1. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A (2010) Cyprinid herpesvirus 3. Emerg Infect Dis 16: 1835-1843.
2. Perelberg A, Ronen A, Hutoran M, Smith Y, Kotler M (2005) Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. Vaccine 23: 3396-3403.
3. Israel:KoVax (2012) http://www.kovax.co.il/products/kv3-vaccine-against-khv/.
4. Cavoy (2012) http://www.drugs.com/vet/cavoy.html.
5. Perelberg A, Ilouze M, Kotler M, Steinitz M (2008) Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). Vaccine 26: 3750-3756.
6. Costes B, Fournier G, Michel B, Delforge C, Raj VS, et al. (2008) Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. J Virol 82: 4955-4964.
7. Haenen O, Way K, Bergmann S, Ariel E (2004) The emergence of koi herpesvirus and its significance to European aquaculture. Bull Eur Assoc Fish Pathol 24: 293-307.
8. Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, et al. (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine 21: 4677-4684.
9. Waltzek TB, Kelley GO, Stone DM, Way K, Hanson L, et al. (2005) Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. J Gen Virol 86: 1659-1667.
10. Yasumoto S, Kuzuya Y, Yasuda M, Yoshimura T, Miyazaki T (2006) Oral immunization of common carp with a liposome vaccine fusing koi herpesvirus antigen. Fish Pathol 41: 141-145.
11. Fuchs W, Fichtner D, Bergmann SM, Mettenleiter TC (2011) Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. Arch Virol 156: 1059-1063.
12. Sommerset I, Krossøy B, Biering E, Frost P (2005) Vaccines for fish in aquaculture. Expert Rev Vaccines 4: 89-101.
13. Aoki T, Hirono I, Kurokawa K, Fukuda H, Nahary R, et al. (2007) Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. J Virol 81: 5058-5065.
14. Davison AJ, Kurobe T, Gatherer D, Cunningham C, Korf I, et al. (2013) Comparative genomics of carp herpesviruses. J Virol 87: 2908-2922.
15. Ouyang P, Rakus K, Boutier M, Reschner A, Leroy B, et al. (2013) The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. Vet Res 44: 53.

16. Amann R, Rohde J, Wulle U, Conlee D, Raue R, et al. (2013) A new rabies vaccine based on a recombinant Orf virus (Parapoxvirus) expressing the rabies virus glycoprotein. J Virol 87: 1618-1630.
17. Emmenegger E, Kurath G (2008) DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. Vaccine 26: 6415-6421.
18. Goodwin A, Merry G, Sadler J (2006) Detection of the herpesviral hematopoietic necrosis disease agent (Cyprinid herpesvirus 2) in moribund and healthy goldfish: validation of a quantitative PCR diagnostic method. Dis Aquat Organ 69: 137-143.
19. Xu J, Zeng L, Zhang H, Zhou Y, Ma J, et al. (2013) Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, *Carassius auratus gibelio* in China. Vet Microbiol 166: 138-144.
20. van Beurden SJ, Engelsma MY, Roozenburg I, Voorbergen-Laarman MA, van Tulden PW, et al. (2012) Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands. Dis Aquat Organ 101: 69-86.
21. Afonso C, Tulman E, Delhon G, Lu Z, Viljoen G, et al. (2006) Genome of crocodilepox virus. J Virol 80: 4978-4991.
22. Neukirch M, Böttcher K, Sumrarn B (1999) Isolation of a virus from koi with altered gills. B Eur Assoc Fish Pat: 221-224.
23. Costes B, Raj VS, Michel B, Fournier G, Thirion M, et al. (2009) The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin. J Virol 83: 2819-2830.
24. Warming S, Costantino N, Jenkins NA, Copeland NG (2005) Simple and highly efficient BAC recombineering using *gal*K selection. Nucleic Acids Res 33: e36-e36.
25. Fournier G, Boutier M, Stalin Raj V, Mast J, Parmentier E, et al. (2012) Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. Vet Res 43: 6.

Discussion and perspectives

Discussion and perspectives

Herpesviruses are double-stranded DNA viruses identified in a wide variety of animals, ranging from oyster to human. They are able to establish persistent and productive infections in immunocompetent hosts despite provoking an efficient immune response. One of the most fascinating strategies is the acquisition of host immunomodulatory genes, to be used against the host during the course of the infection. One of the best examples of such a gene that has been acquired independently by several herpesvirus from their respective hosts is the pleiotropic cytokine Interleukin-10 (IL-10).

Cyprinid herpesvirus 3 (CyHV-3), a member of the family *Alloherpesviridae*, is the causative agent of a lethal disease in common carp and koi. Because of its economic importance and its numerous original biological properties, CyHV-3 became rapidly a very interesting subject for applied and fundamental research. However, to date, there is little information on the roles of individual CyHV-3 genes in the biology of CyHV-3 infection or in its pathogenesis. Moreover, there is a lack of safe and efficacious vaccine for the control of CyHV-3 disease.

In 2007, the whole genome of CyHV-3 has been sequenced by Aoki *et al.* (2007). CyHV-3 genomes are predicted to contain 155 unique, functional protein-coding genes of which eight are duplicated in the terminal repeat (Aoki *et al.*, 2007; Davison *et al.*, 2013). This achievement was a major milestone towards the study of the roles of CyHV-3 genes in pathogenesis. An additional breakthrough was the cloning of the CyHV-3 genome as a stable and infectious bacterial artificial chromosome (BAC), which can be used to produce CyHV-3 recombinants (Costes *et al.*, 2008). These advances provided crucial tools to study the roles of individual CyHV-3 genes. Interestingly, CyHV-3 genome encodes several proteins that could be involved in immune evasion processes, such as, ORF4 and ORF12, which are both predicted to encode for tumor necrosis factor receptor (TNFR) orthologues; ORF16, which encodes for a potential G-protein couple receptor (GPCR); and ORF134, which encodes for an IL-10 orthologue (Aoki *et al.*, 2007). The initial goal of this thesis was to study the roles of ORF134 in the biology of CyHV-3 infection *in vitro* and *in vivo*.

Virally encoded IL-10 orthologues have been identified in several members of the *Poxviridae* family and the *Herpesvirales* order (Hughes, 2002; Kotenko SV, 2001; Slobedman *et al.*, 2009). Up to date, viral IL-10 orthologues (vIL-10s) have been reported for 12 members of the *Herpesviridae* family, for 2 members of the *Alloherpesviridae* family, and for 7 members of the *Poxviridae* family. However, the biology functional of vIL-10s have been described *in vitro* only in 4 members of *Herpesviridae* family (Human cytomegalovirus [HCMV], Rhesus cytomegalovirus [RhCMV], Human HV 4 [EBV], Ovine HV 2 [OvHV2]) and in 1 member of *Poxviridae* family (Orf virus [ORFV]). This has not yet been investigated for members of the *Alloherpesviridae* family. Even more restrictive, the role of vIL-10s has been investigated in the pathogenesis for only one member of the *Herpesviridae* family (RhCMV) and one member of the *Poxviridae* family (ORFV) (Chang & Barry, 2010; Eberhardt *et al.*, 2012; Fleming *et al.*, 2007). In the first experimental chapter of the present thesis, we

described for the first time the roles of a vIL-10 encoded by a member of *Alloherpesviridae* family in the biology of the infection *in vitro* and *in vivo*.

In vitro, transcriptomic analysis demonstrated that CyHV-3 ORF134 is expressed as a spliced E-L gene. This observation is consistent with the results published by Ilouze *et al.* who concluded that ORF134 is an E gene (Ilouze *et al.*, 2012). It is also consistent with the results reported for other vIL-10s which are expressed as E [HCMV (Jenkins *et al.*, 2008; Lockridge *et al.*, 2000); RhCMV (Lockridge *et al.*, 2000)] or L [EBV (Hudson *et al.*, 1985; Miyazaki *et al.*, 1993; Touitou *et al.*, 1996); HCMV (Chang *et al.*, 2004); AngHV-1 (van Beurden *et al.*, 2013)] genes. The expression product of ORF134 is an abundant secreted protein in the supernatant of CyHV-3 infected cells. It is synthesized as a precursor expressing a 17 amino acids (aa) hydrophilic signal peptide at the N-terminus (Signal peptide was predicted using the signalP 4.0 prediction server [http://www.cbs.dtu.dk/services/SignalP/]). This peptide is cleaved during secretion (Kotenko SV, 2001). It is consistent with the results published for other secreted vIL-10s [HCMV (Chang *et al.*, 2004); EBV (Touitou *et al.*, 1996)]. The expression product of ORF134 could act as a functional vIL-10 homologue able to deregulate the immune response of the infected host. This hypothesis is further supported by a recent publication addressing the biological activity of CyHV-3 ORF134 in zebrafish using an artificial model (Sunarto *et al.*, 2012). However, the results of the present study demonstrated that ORF134 is essential neither for viral replication *in vitro* nor for virulence *in vivo*. *In vitro*, multi-step growth assay results revealed that ORF134 is not essential for CyHV-3 replication *in vitro*. This observation is consistent with the results published for HCMV (Dunn *et al.*, 2003), RhCMV (Chang & Barry, 2010) and ORFV (Fleming *et al.*, 1997). *In vivo*, we took advantage of the "CyHV-3 – carp" model of infection to investigate the role of ORF134 vIL-10 in the biology of CyHV-3 infection in its natural host. For viral inoculation, we mimicked natural infection using immersion in infectious water containing a broad range of virus concentration (ranging from 4 to 400 pfu/ml). Study of viral entry into fish demonstrated that the lowest dose tested (4 pfu/ml) is insufficient to induce an initial synchronous infection of all fish (Costes *et al.*, 2009). Consequently, the infection performed with this lowest dose implicates the spread of the virus within the tank from primary infected fish to cohabitant fish that were initially not infected during inoculation. However, none of the doses tested, revealed a significant difference between wild type, ORF134 deleted recombinant and a derived revertant strain despite the different read-outs performed.

In mammals, 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 (Chapter 3, Introduction). vIL-10s have been showed have a broad immunosuppressive spectrum, ranging from a reduction of proinflammatory responses and decreased expression of MHC class I and II genes (Spencer *et al.*, 2002), to inhibition of dendritic cells (DC) maturation, functionality and survival (Chang *et al.*, 2009; Slobedman *et al.*, 2009). Immunostimulatory properties also have been

demonstrated in some vIL-10s (Jaworowski *et al.*, 2009; Logsdon *et al.*, 2011; Spencer *et al.*, 2008). *In vivo*, RhCMV vIL-10 modulated macrophage infiltration into infected tissue, altered kinetics and magnitude of antiviral antibody response and dampened T cell responses after stimulation with viral antigens (Chang & Barry, 2010). Fleming *et al.* observed smaller and less severe lesions in sheep after primary infection infected with the vIL-10 knock-out virus than those observed in sheep infected with the wild type parental or revertant strains (Fleming *et al.*, 2007).

CyHV-3 vIL-10 is the second most abundant protein secreted in the supernatant. It is essential neither for viral replication *in vitro* nor for virulence *in vivo*. While the present study was not able to incriminate a role for ORF134 neither during *in vitro* nor *in vivo* infection, it is still possible that this ORF plays a role that could not be revealed with the experimental setting used. Firstly, it could be possible that ORF134 has a role during the latent infection. However, in CyHV-3 infected carp, ORF134 is highly expressed during acute and reactivation phases, while it is only expressed at a low level during low-temperature induced persistent phase (Sunarto *et al.*, 2012). Secondly, it could be possible that ORF134 has immunomodulatory functions other than those tested in the present study.

CyHV-3 diseases outbreaks usually induced mortality rate between 60-100%. The high mortality induced by CyHV-3 together with its rapid spread explains why soon after its discovery it became an interesting subject for applied science. Many researchers tried to find a proper method to control this lethal disease. Based on the restriction of its replication by temperature, immunization against CyHV-3 was achieved by co-habitation with sick fish for 3-5 days at permissive temperature (22-23°C) and subsequently transferring the exposed carp to a non-permissive temperature (30°C). Challenge performed on the surviving fish showed partial protection (mortality rate of 39% compare to 82% for the control group). The disadvantages of this method have been discussed previously (Chapter 2, Experimental section). Vaccination is the best method to control CyHV-3 disease. Recently, a live attenuated vaccine developed by *in vitro* serial passages and treatment with UV irradiation has been manufactured by KoVax Ltd. (Jerusalem, Israel) and is available for immersion vaccination of common and koi carp in Israel (Israel:KoVax, 2012; Perelberg *et al.*, 2005; Ronen *et al.*, 2003). This vaccine has several disadvantages. Firstly, the determinism of the attenuation is unknown and it seems insufficient for vaccination of highly sensitive small fish. This explains why the temporary commercialization of this vaccine in the USA was restricted to fish weighing more than 100 g (Norvartis Animal Health). Secondly, the duration of the protection conferred by the vaccine has not been established. Thirdly, the protection level of this vaccine never reaches 100% (about 80%). In the second study, we developed a good attenuated candidate vaccine by deletion of specific ORFs.

Since we succeeded to cloning CyHV-3 genome as an infection artificial chromosome (BAC) in 2008, we produced several recombinants deleted for specific ORFs. None of the selected ORFs induced sufficient attenuation when tested *in vivo*. However, during the production of a recombinant deleted for ORF134 (Chapter 1, Experimental section), an additional unexpected mutation was obtained. This strain which was deleted for ORF56-57 and ORF134 exhibited a highly attenuated

phenotype even when it was tested on young carp as small as 1 g. To identify the determinism of the attenuation of this strain, ORF56-57 double deleted recombinants and independent ORF56-57-134 triple deleted recombinants were produced and tested *in vivo*. The obtained results demonstrated that the ORF56-57 deletion that occurred unexpectedly in the mutant strain is responsible for the highly attenuation observed. Moreover, we confirmed that the FL BAC ORF56-57 Del strain can induce a protective immune response as demonstrated by challenge with a wild type strain expressing luciferase as a reporter gene. All together these results demonstrated that the CyHV-3 ORF56-57 Del strain is a good candidate vaccine.

In this study, the component(s) of the adaptive immune system conferring protection following vaccination with the FL BAC ORF56-57 Del strain was not investigated. However, previous work on attenuated CyHV-3 vaccine has showed good correlation between levels of protection induced and the level of specific antibodies against CyHV-3. Nevertheless, immunized fish, even those in which antibodies were no longer detectable were resistant to a lethal challenge; possibly because of the subsequent rapid response of B and T memory cells to antigen re-stimulation (Perelberg *et al*., 2008). Antibodies from CyHV-3 vaccinated fish also showed high neutralization against CyHV-3 antigens *in vitro* by using neutralizing assays (Perelberg *et al*., 2008). This study suggests that neutralizing antibodies are likely to play a key role in the immune protection induced by attenuated vaccines against CyHV-3. We observed sterile protection in the skin by using IVIS at 2-8 days post challenge with the FL BAC revertant 136 LUC strain (Chapter 2, Experimental section). Components of the carp mucosal immune response, including immunoglobulins could be responsible for this apparent sterile protection. However, the exact immune mechanisms involved in the protection induced by CyHV-3 ORF56-57 Del strain still need be further investigate in the future.

Deletion of ORF56 and ORF57 induces a strong attenuation *in vivo* without preventing viral replication *in vitro*. Further studies are required to determine whether deletions of ORF56 and/or ORF57 are responsible for the safety/efficacy profile. CyHV-3 ORF56 is a 2571 bp ORF encoding a 856 amino acid product, while ORF57 encode a 473 amino acid product abundantly present in CyHV-3 virions (Michel *et al.*, 2010). In the ORF56-57 Del strain, most of ORF56 has been deleted with only the last 296 bp left. Taking into account alternative ATG, the residual sequence of ORF56 would only lead to the expression of the last 77 aa of the original protein. There is high chance that such a truncated protein does not yield to a functional protein. In contrast, the deletion of ORF57 was compatible with the expression of a truncated protein encompassing the last 333 aa of the original protein. To determine whether such truncated protein was expressed, CyHV-3 ORF56-57 Del virions were purified and analyzed by 2D-LC-MS/MS (Michel *et al.*, 2010). pORF57 was detected in the FL BAC excised strain, but not in the FL BAC excised double deleted strain and FL BAC excised triple deleted strain (data not shown). The obtained results demonstrated that the remaining sequence of ORF57 does not encode a truncated protein. In order to investigate whether the ORF56-57 deletion affects the structure of CyHV-3 virion, semi-purified viral particles of FL BAC excised strain, FL

BAC excised double deleted strain, FL BAC excised triple deleted strain were submitted to electron microscopy examination. The double deleted, the triple deleted and the wild type virions exhibited structure that could not be discriminated (data not shown).

Compared to human and domestic animals, immunization of fish against diseases still has limited applications. One of the reasons explaining this lacuna is the immunization method. Immunization of fish by injectable vaccines is effective but not yet practical on a large scale. For this reason many researchers concentrated on development of oral immunization. This method has been used against bacterial diseases such as vibriosis, columnaris, furunculosis, and red mouth (Anderson, 1974; Romalde *et al.*, 2004), as well as some viral diseases, such as infectious pancreatic necrosis virus (IPNV) (de las Heras *et al.*, 2010). However, the administration of the antigens by the oral route is complicated by problems related to preparation and/or distribution. In contrast to injection and oral immunization, immersion vaccination has at least two advantages: (i) it is fast, thousands of fish can be vaccinated in few hours; (ii) it is easy to perform, the vaccine is directly dropped into the tanks. The ORF56-57 Del strain that we produced in the second experimental chapter can be used to vaccinate fish by immersion.

This vaccine candidate developed has several advantages. (i) It is simple to use, the fish just need to be immersed into the solution vaccine for two hours; (ii) it is safe, even for fish as small as 1 g at the dose of 400 pfu/mL (high dose); (iii) it is efficacious, vaccination performed at 40 pfu/mL induced 100% survival; (iv) experiments performed by Dr M. Boutier demonstrated that the vaccine strain has a reduced ability to spread from vaccinated fish to naïve cohabitant (data not shown); (v) the determinism of the attenuation is known and the risk of reversion can be excluded.

To determine precisely which ORF contribute to the attenuation of ORF56-57 Del strain. Deletion of ORF56 or ORF57 will be produced by using BAC cloning technologies. To exclude the potential polar effect of the deletion, we will also produce antibodies against ORF56 and ORF57 by using DNA immunization as described elsewhere (Lemaire *et al.*, 2011). In order to study the properties of the vaccine candidate to replicate into vaccinated fish, we plan to produce a FL BAC revertant ORF56-57 Del strain expressing LUC gene as a reporter. By using IVIS analysis, this strain will allow us to study the tropism of the vaccine strain compared to a wild type strain (also encoding a LUC cassette). Moreover, it will also allow us to study the portal of entry of the vaccine and its putative spreading to naïve cohabitant fish. Once the vaccine candidate will be fully characterized, we plan to test its potential as an expression vector by developing recombinant derived strain expressing glycoprotein G of spring viraemia of carp (SVC) as a transgene.

The perspectives of the present work are also fundamental. We plan to unravel the roles of ORF56 and/ or ORF57 in the biology of CyHV-3 infection both *in vitro* and *in vivo*. When studying the structural proteome of the ORF56-57 double deleted strain (data not shown), we discovered an interesting link between ORF57 and ORF34. Indeed, the absence of pORF57 in the structural proteome systematically led to the absence of pORF34. This ORF encodes an unclassified structural

protein (Michel *et al.*, 2010), without any predicted transmembrane domain but with a predicted signal peptide (Davison *et al.*, 2013). The study of the interaction between pORF57 and pORF34, as well as potential additional modifications in the structural proteome, could reveal interesting clues about the role of pORF57 in the viral morphogenesis, and perhaps an unexpected role of pORF34 in the observed attenuated phenotype.

In conclusion, in the present thesis we addressed for the first time the role of a viral IL-10 encoded by a member of the *Alloherpesviridae* family both *in vitro* and *in vivo*. During the course of this first study, an unexpected event was discovered and became the beginning of the work for the second experimental chapter. The characterization of a triple deleted recombinant obtained by chance lead to the identification of the ORF56-57 loci as perfect candidate loci for production of attenuated recombinant CyHV-3 vaccines. This thesis illustrates the fact that while science must always be rational and critical, part of its progress relies on chance.

Summary

Summary

The common carp is one of the most important freshwater fish species in aquaculture, and its colourful subspecies koi is grown for personal pleasure and competitive exhibitions. Both two subspecies are economically important. In the late 1990s, a highly contagious and lethal pathogen called koi herpesvirus (KHV) or cyprinid herpesvirus 3 (CyHV-3) began to cause severe financial losses in these two carp industries worldwide. In 2005, CyHV-3 has been classified in the *Alloherpesviridae* family of the order *Herpesvirales.* Because of its economic importance and its numerous original biological properties, CyHV-3 became rapidly an attractive subject for applied and fundamental research. However, to date, there is a little information on the roles of individual CyHV-3 genes in the biology of CyHV-3 infection or its pathogenesis. Moreover, there is a lack of safe and efficacious vaccine for the control of CyHV-3 disease. The goal of this thesis was to study the roles of CyHV-3 ORF134 encoding an IL-10 homologue in the biology of the infection.

CyHV-3 ORF134 has been predicted to contain an 84 bp intron flanked by 2 exons encoding together a 179 amino acid product. Transcriptomic analyses reveal that ORF134 is expressed as a spliced early-late gene. The identification of the CyHV-3 secretome was achieved using 2D-LC MS/MS proteomic approach. This method led to the identification of 5 viral and 46 cellular proteins in concentrated infected cell culture supernatant. CyHV-3 ORF12 and ORF134 were amongst the most abundant proteins detected. To investigate the roles of ORF134 in the biological of the infection, a strain deleted for ORF134 and a derived revertant strain were produced by using BAC cloning and prokaryotic recombination technologies. Comparison of these strains demonstrated that CyHV-3 ORF134 does not contribute significantly to viral growth *in vitro* or to virulence *in vivo* in the present laboratory setting. The present study addressed for the first time the *in vivo* role of a vIL-10 encoded by a member of the family *Alloherpesviridae.* This study has been published in *Veterinary Research*.

During the course of the first study, we obtained an unexpected recombination event while we were reconstituting infectious virus from mutated BAC plasmids. To generate a revertant ORF134 Del *gal*K strain, CCB cells were co-transfected with the FL BAC ORF134 Del *gal*K plasmid and the pGEMT-TK vector to remove the BAC cassette inserted in the ORF55 locus (encoding thymidine kinase). One of the clones obtained had an unexpected recombination leading to the deletion of ORF56 and ORF57 in addition to the expected deletion of ORF134. Unexpectedly, this triple deleted strain replicated efficiently *in vitro,* exhibited an attenuated phenotype *in vivo* and was proved to confer in a dose dependent manner an immune protection against a lethal challenge. The goal of the second experimental chapter was to investigate the role of the ORF56-57 and ORF134 deletions in the observed safety/efficacy profile of the triple deleted recombinant. To reach this goal, a collection of recombinant strains were produced using BAC cloning technologies, characterized and tested *in vivo* for their safety/efficacy profile. The results obtained demonstrated that the ORF56-57 deletion is responsible for the phenotype observed and that ORF134 deletion does not contribute to this

phenotype significantly. Finally, the immune protection conferred by ORF56-57 deleted recombinant was investigated by challenging immunized fish with a wild type strain expressing luciferase as a reporter gene. *In vivo imaging system* (IVIS) analyses of immunized and challenged fish demonstrated that the immune response induced by the ORF56-57 deleted strain was able to prevent subclinical infection of the challenge strain.

In conclusion, the present thesis addressed both fundamental and applied aspects of CyHV-3. For the first time, it investigated *in vivo* the roles of a viral IL-10 homologue encoded by a member of the family *Alloherpesviridae*. Importantly, it identified the ORF56-57 loci as target for production of safe and efficacious attenuated recombinant vaccines.

References

A

Ackermann, M. (2004). Herpesviruses: a brief overview. *Methods Mol Biol* **256**, 199-219.

Adamek, M., Rakus, K. L., Chyb, J., Brogden, G., Huebner, A., Irnazarow, I. & Steinhagen, D. (2012). Interferon type I responses to virus infections in carp cells: *In vitro* studies on Cyprinid herpesvirus 3 and *Rhabdovirus carpio* infections. *Fish Shellfish Immunol* **33**, 482-493.

Adamek, M., Syakuri, H., Harris, S., Rakus, K. L., Brogden, G., Matras, M., Irnazarow, I. & Steinhagen, D. (2013). Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.). *Vet Microbiol* **162**, 456-470.

Afonso, C., Tulman, E., Delhon, G., Lu, Z., Viljoen, G., Wallace, D., Kutish, G. & Rock, D. (2006). Genome of crocodilepox virus. *J Virol* **80**, 4978-4991.

Allavena, P., Piemonti, L., Longoni, D., Bernasconi, S., Stoppacciaro, A., Ruco, L. & Mantovani, A. (1998). IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. *Eur J Immunol* **28**, 359-369.

Amann, R., Rohde, J., Wulle, U., Conlee, D., Raue, R., Martinon, O. & Rziha, H.-J. (2013). A new rabies vaccine based on a recombinant Orf virus (Parapoxvirus) expressing the rabies virus glycoprotein. *J Virol* **87**, 1618-1630.

Anderson, D. P. (1974). Fish immunology. Diseases of fishes. Edited by Dr. Stanislas F.Snieszko and Dr.Herbert R. Axelrod. Published by T.F.H. Publications.

Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H. & other authors (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J Virol* **81**, 5058-5065.

Aoki, T., Takano, T., Unajak, S., Takagi, M., Kim, Y. R., Park, S. B., Kondo, H., Hirono, I., Saito-Taki, T. & other authors (2011). Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus. *Comp Immunol Microbiol Infect Dis* **34**, 209-216.

Arrand, J. R., Rymo, L., Walsh, J. E., Björck, E., Lindahl, T. & Griffin, B. E. (1981). Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping restriction endonuclease fragments. *Nucleic Acids Res* **9**, 2999-3014.

Avarre, J. C., Madeira, J. P., Santika, A., Zainun, Z., Baud, M., Cabon, J., Caruso, D., Castric, J., Bigarré, L. & other authors (2011). Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable number of tandem repeats analysis. *J Virol Methods* **173**, 320-327.

Avdic, S., Cao, J. Z., Cheung, A. K., Abendroth, A. & Slobedman, B. (2011). Viral interleukin-10 expressed by human cytomegalovirus during the latent phase of infection modulates latently infected myeloid cell differentiation. *J Virol* **85**, 7465-7471.

Avdic, S., Cao, J. Z., McSharry, B. P., Clancy, L. E., Brown, R., Steain, M., Gottlieb, D. J., Abendroth, A. & Slobedman, B. (2013). Human cytomegalovirus interleukin-10 polarizes monocytes toward a deactivated M2c phenotype to repress host immune responses. *J Virol* **87**, 10273-10282.

B

Ballestas, M. E. & Kaye, K. M. (2001). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. *J Virol* **75**, 3250-3258.

Balon, E. K. (1995). Origin and domestication of the wild carp, Cyprinus carpio: from Roman gourmets to the swimming flowers. *Aquaculture* **129**, 3-48.

Balon, E. K. (2006). The oldest domesticated fishes, and the consequences of an epigenetic dichotomy in fish culture. *J Ichthyol Aquat Biol* **11**, 47-86.

Banchereau, J., Pascual, V. & O'Garra, A. (2012). From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat Immunol* **13**, 925-931.

Bartlett, N. W., Dumoutier, L., Renauld, J. C., Kotenko, S. V., McVey, C. E., Lee, H. J. & Smith, G. L. (2004). A new member of the interleukin 10-related cytokine family encoded by a poxvirus. *J Virol* **85**, 1401-1412.

Bergmann, S. M. & Kempter, J. (2011). Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods. *Bull Eur Assoc Fish Pathol* **31**, 92-100.

Bergmann, S. M., Lutze, P., Schütze, H., Fischer, U., Dauber, M., Fichtner, D. & Kempter, J. (2010). Goldfish (*Carassius auratus auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *Bull Eur Assoc Fish Pathol* **30**, 74-84.

Bergmann, S. M., Schütze, H., Fischer, U., Fichtner, D., Riechardt, M., Meyer, K., Schrudde, D. & Kempter, J. (2009). Detection KHV genome in apparently health fish. *Bull Eur Assoc Fish Pathol* **29**, 145-152.

Bergmann, S. M., Sadowski, J., Kielpinski, M., Bartlomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M. & Kempter, J. (2010). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *J Fish Dis* **33**, 267-272.

Bigarre, L., Baud, M., Cabon, J., Antychowicz, J., Bergmann, S. M., Engelsma, M., Pozet, F., Reichert, M. & Castric, J. (2009). Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR. *J Virol Methods* **158**, 51-57.

Brady, M. T., MacDonald, A. J., Rowan, A. G. & Mills, K. H. G. (2003). Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur J Immunol* **33**, 3448-3457.

Bratke, K. A. & McLysaght, A. (2008). Identification of multiple independent horizontal gene transfers into poxviruses using a comparative genomics approach. *BMC Evol Biol* **8**, 67.

Bretzinger, A., Fischer-Scherl, T., Oumouna, M., Hoffmann, R. & Truyen, U. (1999). Mass mortalities in Koi carp, *Cyprinus carpio*, associated with gill and skin disease. *Bull Eur Assoc Fish Pathol* **19**, 182-185.

Brockman, M. A., Kwon, D. S., Tighe, D. P., Pavlik, D. F., Rosato, P. C., Sela, J., Porichis, F., Le Gall, S., Waring, M. T. & other authors (2009). IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. *Blood* **114**, 346-356.

Brodeur, N. D. & Spencer, J. V. (2010). Antibodies to human IL-10 neutralize ebvIL-10-mediated cytokine suppression but have no effect on cmvIL-10 activity. *Virus Res* **153**, 265-268.

Browne, H., Bell, S., Minson, T. & Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**, 4311-4316.

Brunovskis, P. & Kung, H. J. (1995). Retrotransposition and herpesvirus evolution. *Virus Genes* **11**, 259-270.

Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B. C., Green, P. J., Jain, N., Isaacs, G. & Morgan, R. W. (2006). Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. *J Virol* **80**, 8778-8786.

C

Cai, G., Kastelein, R. A. & Hunter, C. A. (1999). IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur J Immunol* **29**, 2658-2665.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci U S A* **102**, 5570-5575.

Cardin, R. D., Schaefer, G. C., Allen, J. R., Davis-Poynter, N. J. & Farrell, H. E. (2009). The M33 chemokine receptor homolog of murine cytomegalovirus exhibits a differential tissue-specific role during in vivo replication and latency. *J Virol* **83**, 7590-7601.

Carson, W. E., Lindemann, M. J., Baiocchi, R., Linett, M., Tan, J. C., Chou, C.-C., Narula, S. & Caligiuri, M. (1995). The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood* **85**, 3577-3585.

Cassatella, M. A., Meda, L., Bonora, S., Ceska, M. & Constantin, G. (1993). Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide. *J Exp Med* **178**, 2207-2211.

Cavoy (2012). http://www.drugs.com/vet/cavoy.html.

Chan, A., Baird, M., Mercer, A. A. & Fleming, S. B. (2006). Maturation and function of human dendritic cells are inhibited by orf virus-encoded interleukin-10. *J Gen Virol* **87**, 3177-3181.

Chang, W. L. & Barry, P. A. (2010). Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity. *Proc Natl Acad Sci U S A* **107**, 22647-22652.

Chang, W. L. W., Baumgarth, N., Yu, D. & Barry, P. A. (2004). Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J Virol* **78**, 8720-8731.

Chang, W. L., Barry, P. A., Szubin, R., Wang, D. & Baumgarth, N. (2009). Human cytomegalovirus suppresses type I interferon secretion by plasmacytoid dendritic cells through its interleukin 10 homolog. *Virology* **390**, 330-337.

Cheeran, M. C. J., Hu, S., Sheng, W. S., Peterson, P. K. & Lokensgard, J. R. (2003). CXCL10 production from cytomegalovirus-stimulated microglia is regulated by both human and viral interleukin-10. *J Virol* **77**, 4502-4515.

Chernoff, A. E., Granowitz, E. V., Shapiro, L., Vannier, E., Lonnemann, G., Angel, J. B., Kennedy, J. S., Rabson, A. R., Wolff, S. M. & other authors (1995). A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J Immunol* **154**, 5492-5499.

Cheung, A. K., Gottlieb, D. J., Plachter, B., Pepperl-Klindworth, S., Avdic, S., Cunningham, A. L., Abendroth, A. & Slobedman, B. (2009). The role of the human cytomegalovirus UL111A gene in down-regulating $CD4^+$ T-cell recognition of latently infected cells: implications for virus elimination during latency. *Blood* **114**, 4128-4137.

Costes, B., Ruiz-Arguello, M. B., Bryant, N. A., Alcami, A. & Vanderplasschen, A. (2005). Both soluble and membrane-anchored forms of Felid herpesvirus 1 glycoprotein G function as a broad-spectrum chemokine-binding protein. *J Gen Virol* **86**, 3209-3214.

Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., Gillet, L., Drion, P., Body, A. & other authors (2008). Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *J Virol* **82**, 4955-4964.

Costes, B., Raj, V. S., Michel, B., Fournier, G., Thirion, M., Gillet, L., Mast, J., Lieffrig, F., Bremont, M. & other authors (2009). The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin. *J Virol* **83**, 2819-2830.

Couper, K. N., Blount, D. G. & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *J Immunol* **180**, 5771-5777.

D

D'Andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M. & Trinchieri, G. (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* **178**, 1041-1048.

Darlington, R. W. & Moss, L. H., 3rd (1968). Herpesvirus envelopment. *J Virol* **2**, 48-55.

Davidovich, M., Dishon, A., Ilouze, M. & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Arch Virol* **152**, 1541-1546.

Davison, A. J. (1984). Structure of the genome termini of varicella-zoster virus. *J Gen Virol* **65 (Pt 11)**, 1969-1977.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A. J. (2002). Evolution of the herpesviruses. *Vet Microbiol* **86**, 69-88.

Davison, A. J. & Stow, N. D. (2005). New genes from old: redeployment of dUTPase by herpesviruses. *J Virol* **79**, 12880-12892.

Davison, A., Holton, M., Dolan, A., Dargan, D., Gatherer, D. & Hayward, G. (2013). Comparative genomics of primate cytomegaloviruses. In Cytomegaloviruses: from molecular pathogenesis to intervention. Edited by Matthias J Reddehase.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., Le Deuff, R. M. & Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86**, 41-53.

Davison, A. J., Kurobe, T., Gatherer, D., Cunningham, C., Korf, I., Fukuda, H., Hedrick, R. P. & Waltzek, T. B. (2013). Comparative genomics of carp herpesviruses. *J Virol* **87**, 2908-2922.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J. & other authors (2009). The order Herpesvirales. *Arch Virol* **154**, 171-177.

de las Heras, A. I., Rodríguez Saint-Jean, S. & Pérez-Prieto, S. I. (2010). Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish. *Fish Shellfish Immunol* **28**, 562-570.

de Lemos Rieper, C., Galle, P., Pedersen, B. K. & Hansen, M. B. (2011). Characterization of specific antibodies against cytomegalovirus (CMV)-encoded interleukin 10 produced by 28% of CMV-seropositive blood donors. *J Gen Virol* **92**, 1508-1518.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. & de Vries, J. E. (1991a). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174**, 1209-1220.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. & other authors (1991b). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* **174**, 915-924.

Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F. & Banchereau, J. (1992). Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* **175**, 671-682.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M. G., Biagiotti, R. & Romagnani, S. (1993). Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* **150**, 353-360.

Delhon, G., Tulman, E., Afonso, C., Lu, Z., De la Concha-Bermejillo, A., Lehmkuhl, H., Piccone, M., Kutish, G. & Rock, D. (2004). Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. *J Virol* **78**, 168-177.

Demangel, C., Bertolino, P. & Britton, W. J. (2002). Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur J Immunol* **32**, 994-1002.

Denham K, L. (2003). Koi herpesvirus in wild fish. *Vet Rec* **153**, 507.

Dewals, B., Boudry, C., Gillet, L., Markine-Goriaynoff, N., De Leval, L., Haig, D. & Vanderplasschen, A. (2006). Cloning of the genome of Alcelaphine herpesvirus 1 as an infectious and pathogenic bacterial artificial chromosome. *J Gen Virol* **87**, 509-517.

Díaz-San Segundo, F., Rodríguez-Calvo, T., de Avila, A. & Sevilla, N. (2009). Immunosuppression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. *PLoS One* **4**, e5659.

Dimmock, N. J., Easton, A. J. & Leppard, K. N. (2007). Towards a definition of a virus. In *Introduction to modern virology*, 6 edn, pp. 3 - 17. Edited by N. J. Dimmock, A. J. Easton & K. N. Leppard. Oxford: Wiley-Blackwel.

Ding, Y., Qin, L., Kotenko, S. V., Pestka, S. & Bromberg, J. S. (2000). A single amino acid determines the immunostimulatory activity of interleukin 10. *J Exp Med* **191**, 213-224.

Ding, Y., Qin, L., Zamarin, D., Kotenko, S. V., Pestka, S., Moore, K. W. & Bromberg, J. S. (2001). Differential IL-10R1 expression plays a critical role in IL-10-mediated immune regulation. *J Immunol* **167**, 6884-6892.

Dishon, A., Davidovich, M., Ilouze, M. & Kotler, M. (2007). Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. *J Virol* **81**, 4828-4836.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S. & Kotler, M. (2005). Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Appl Environ Microbiol* **71**, 7285-7291.

Dixon, P. F., Joiner, C. L., Way, K., Reese, R. A., Jeney, G. & Jeney, Z. (2009). Comparison of the resistance of selected families of common carp, Cyprinus carpio L., to koi herpesvirus: preliminary study. *J Fish Dis* **32**, 1035-1039.

Dong, C., Li, X., Weng, S., Xie, S. & He, J. (2013). Emergence of fatal European genotype CyHV-3/KHV in mainland China. *Veterinary Microbiology* **162**, 239-244.

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q. & He, J. (2011). Characterization of a new cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid herpesvirus 3 in China. *Virus Res* **161**, 140-149.

Drummer, H. E., Studdert, M. J. & Crabb, B. S. (1998). Equine herpesvirus-4 glycoprotein G is secreted as a disulphide-linked homodimer and is present as two homodimeric species in the virion. *J Gen Virol* **79 (Pt 5)**, 1205-1213.

Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7**, 214.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Zhu, H. & Liu, F. (2003). Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* **100**, 14223-14228.

E

Eberhardt, M. K., Chang, W. L., Logsdon, N. J., Yue, Y., Walter, M. R. & Barry, P. A. (2012). Host immune responses to a viral immune modulating protein: immunogenicity of viral interleukin-10 in rhesus cytomegalovirus-infected rhesus macaques. *PLoS One* **7**, e37931.

Eberhardt, M. K., Deshpande, A., Chang, W. W., Barthold, S. W., Walter, M. R. & Barry, P. A. (2013). Vaccination against a virus-encoded cytokine significantly restricts viral challenge. *J Virol* **87**, 11323-11331.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-1797.

Eide, K., Miller-Morgan, T., Heidel, J., Bildfell, R. & Jin, L. (2011). Results of total DNA measurement in koi tissue by Koi Herpesvirus real-time PCR. *J Virol Methods* **172**, 81-84.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., LaPatra, S., Watson, G. & Jin, L. (2011). Investigation of koi herpesvirus latency in koi. *J Virol* **85**, 4954-4962.

El-Matbouli, M. & Soliman, H. (2011). Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naive common carp by cohabitation. *Res Vet Sci* **90**, 536-539.

El-Matbouli, M., Saleh, M. & Soliman, H. (2007). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (*Cyprinus carpio koi*). *Vet Rec* **161**, 792-793.

Emmenegger, E. & Kurath, G. (2008). DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. *Vaccine* **26**, 6415-6421.

F

Fabian, M., Baumer, A. & Steinhagen, D. (2012). Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *J Fish Dis* **36**, 505-514.

FAO (2012). Statistics and Information Service of the Fisheries and Aquaculture Department: Fishery and Aquaculture Statistics 2010. Rome: FAO; 2012.

Fiorentino, D. F., Bond, M. W. & Mosmann, T. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* **170**, 2081-2095.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147**, 3815-3822.

Fleming, S. B., McCaughan, C. A., Andrews, A. E., Nash, A. D. & Mercer, A. A. (1997). A homolog of interleukin-10 is encoded by the poxvirus orf virus. *J Virol* **71**, 4857-4861.

Fleming, S. B., Haig, D. M., Nettleton, P., Reid, H. W., McCaughan, C. A., Wise, L. M. & Mercer, A. (2000). Sequence and functional analysis of a homolog of interleukin-10 encoded by the parapoxvirus orf virus. *Virus Genes* **21**, 85-95.

Fleming, S. B., Anderson, I. E., Thomson, J., Deane, D. L., McInnes, C. J., McCaughan, C. A., Mercer, A. A. & Haig, D. M. (2007). Infection with recombinant orf viruses demonstrates that the viral interleukin-10 is a virulence factor. *J Gen Virol* **88**, 1922-1927.

Flint, S., Racaniello, V. R., Enquist, L. W., Skalka, A. M. & Krug, R. M. (2000). *Virology : molecular biology, pathogenesis, and control*, First edn edn. Washington, D. C: ASM press.

Fournier, G., Boutier, M., Stalin Raj, V., Mast, J., Parmentier, E., Vanderwalle, P., Peeters, D., Lieffrig, F., Farnir, F. & other authors (2012). Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Vet Res* **43**, 6.

Franken, M., Devergne, O., Rosenzweig, M., Annis, B., Kieff, E. & Wang, F. (1996). Comparative analysis identifies conserved tumor necrosis factor receptor-associated factor 3 binding sites in the human and simian Epstein-Barr virus oncogene LMP1. *J Virol* **70**, 7819-7826.

Fuchs, W., Fichtner, D., Bergmann, S. M. & Mettenleiter, T. C. (2011). Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. *Arch Virol* **156**, 1059-1063.

G

Gad, H. H., Dellgren, C., Hamming, O. J., Vends, S., Paludan, S. R. & Hartmann, R. (2009). Interferon-λ is functionally an interferon but structurally related to the interleukin-10 family. *J Biol Chem* **284**, 20869-20875.

Garber, D. A., Beverley, S. M. & Coen, D. M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* **197**, 459-462.

Garver, K. A., Al-Hussinee, L., Hawley, L. M., Schroeder, T., Edes, S., LePage, V., Contador, E., Russell, S., Lord, S. & other authors (2010). Mass mortality associated with koi herpesvirus in wild common carp in Canada. *J Wildl Dis* **46**, 1242-1251.

Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H. & Hedrick, R. P. (2004). Concentrations of a *Koi herpesvirus* (KHV) in tissues of experimentally infected Cyprinus carpio koi as assessed by real-time TaqMan PCR. *Dis Aquat Organ* **60**, 179-187.

Go, N. F., Castle, B. E., Barrett, R., Kastelein, R., Dang, W., Mosmann, T. R., Moore, K. W. & Howard, M. (1990). Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med* **172**, 1625-1631.

Goodwin, A., Merry, G. & Sadler, J. (2006). Detection of the herpesviral hematopoietic necrosis disease agent (Cyprinid herpesvirus 2) in moribund and healthy goldfish: validation of a quantitative PCR diagnostic method. *Dis Aquat Organ* **69**, 137-143.

Gotesman, M., Soliman, H. & El-Matbouli, M. (2013). Antibody screening identifies 78 putative host proteins involved in Cyprinid herpesvirus 3 infection or propagation in common carp, *Cyprinus carpio* L. *J Fish Dis* **36**, 721–733.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N. & Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**, 3675-3684.

Grimmett, S. G., Warg, J. V., Getchell, R. G., Johnson, D. J. & Bowser, P. R. (2006). An unusual koi herpesvirus associated with a mortality event of common carp Cyprinus carpio in New York State, USA. *J Wildl Dis* **42**, 658-662.

H

Haenen, O. L. M., Way, K., Bergmann, S.M., and Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bull Eur Assoc Fish Pathol* **24**, 293-307.

Haenen, O., Way, K., Bergmann, S. & Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bull Eur Assoc Fish Pathol* **24**, 293-307.

Haenen, O. L. M., Dijkstra, S. G., Van Tulden, P. W., Davidse, A., Van Nieuwstadt, A. P., Wagenaar, F. & Wellenberg, G. J. (2002). Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, Anguilla anguilla in the Netherlands since 1996. *Bull Eur Assoc Fish Pathol* **22**, 247-257.

Haig, D. M., Thomson, J., McInnes, C., McCaughan, C., Imlach, W., Mercer, A. & Fleming, S. (2002a). Orf virus immuno-modulation and the host immune response. *Vet Immunol Immunopathol* **87**, 395-399.

Haig, D. M., Thomson, J., McInnes, C. J., Deane, D. L., Anderson, I. E., McCaughan, C. A., Imlach, W., Mercer, A. A., Howard, C. J. & other authors (2002b). A comparison of the anti-inflammatory and immuno-stimulatory activities of orf virus and ovine interleukin-10. *Virus Res* **90**, 303-316.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houlès, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J. F. & other authors (2002). Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell *trans*-infection. *Immunity* **17**, 653-664.

Han, J. E., Kim, J. H., Renault, T., Jr., C., Shin, S. P., Jun, J. W. & Park, S. C. (2013). Identifying the viral genes encoding envelope glycoproteins for differentiation of Cyprinid herpesvirus 3 isolates. *Viruses* **5**, 568-576.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Hart, P. H., Hunt, E. K., Bonder, C. S., Watson, C. J. & Finlay-Jones, J. J. (1996). Regulation of surface and soluble TNF receptor expression on human monocytes and synovial fluid macrophages by IL-4 and IL-10. *J Immunol* **157**, 3672-3680.

Hautaniemi, M., Ueda, N., Tuimala, J., Mercer, A. A., Lahdenperä, J. & McInnes, C. J. (2010). The genome of pseudocowpoxvirus: comparison of a reindeer isolate and a reference strain. *J Gen Virol* **91**, 1560-1576.

Hedrick, R. P. (1996). Movement of pathogens with the international trade of live fish: problems and solutions. *Rev Sci Tech* **15**, 523-531.

Hedrick, R. P., Waltzek, T. B. & McDowell, T. S. (2006). Susceptibility of koi carp, common carp, goldfish, and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpes virus-3. *J Aquat Anim Health* **18**, 26-34.

Hedrick, R., Gilad, O., Yun, S., Spangenberg, J., Marty, G., Nordhausen, R., Kebus, M., Bercovier, H. & Eldar, A. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J Aquat Anim Health* **12**, 44-57.

Hedrick R, P., McDowell T, S., Groff J, M., Yun S & Wingfield W, H. (1991). Isolation of an epitheliotropic herpesvirus from white sturgeon *Acipenser transmontanus*. *Dis Aquat Org* **11**, 49-56.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**, 8-19.

Honess, R. W. & Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc Natl Acad Sci U S A* **72**, 1276-1280.

Honjo, M. N., Minamoto, T. & Kawabata, Z. (2012). Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds. *Vet Microbiol* **155**, 183-190.

Honjo, M. N., Minamoto, T., Matsui, K., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2010). Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus. *Appl Environ Microbiol* **76**, 161-168.

Hsu, D. H., de Waal Malefyt, R., Fiorentino, D. F., Dang, M. N., Vieira, P., de Vries, J., Spits, H., Mosmann, T. R. & Moore, K. W. (1990). Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. *Science* **250**, 830-832.

Hudson, G. S., Bankier, A. T., Satchwell, S. C. & Barrell, B. G. (1985). The short unique region of the B95-8 Epstein-Barr virus genome. *Virology* **147**, 81-98.

Hughes, A. L. (2002). Origin and evolution of viral interleukin-10 and other DNA virus genes with vertebrate homologues. *J Mol Evol* **54**, 90-101.

Hwu, P., Du, M. X., Lapointe, R., Taylor, M. W. & Young, H. A. (2000). Indoleamine 2, 3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol* **164**, 3596-3599.

I

Ilouze, M., Dishon, A. & Kotler, M. (2012). Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. *Virus Res* **169**, 98-106.

Ilouze, M., Dishon, A. & Kotler, M. (2012). Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature. *Virus Res* **169**, 289-295.

Ilouze, M., Dishon, A., Kahan, T. & Kotler, M. (2006). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Lett* **580**, 4473-4478.

Ilouze, M., Davidovich, M., Diamant, A., Kotler, M. & Dishon, A. (2011). The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: A review. *Ecol Res* **26**, 885-892.

Imlach, W., McCaughan, C. A., Mercer, A. A., Haig, D. & Fleming, S. B. (2002). Orf virus-encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits cytokine synthesis in murine peritoneal macrophages. *J Gen Virol* **83**, 1049-1058.

Isfort, R., Jones, D., Kost, R., Witter, R. & Kung, H. J. (1992). Retrovirus insertion into herpesvirus *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* **89**, 991-995.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J. & Mann, M. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol Cell Proteomics* **4**, 1265-1272.

Israel:KoVax (2012). http://www.kovax.co.il/products/kv3-vaccine-against-khv/.

Ito, T., Sano, M., Kurita, J., Yuasa, K. & Iida, T. (2007). Carp larvae are not susceptible to Koi Herpesvirus. *Fish Pathol* **42**, 107-109.

J

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-457.

Jaworowski, A., Cheng, W. J., Westhorpe, C. L., Abendroth, A., Crowe, S. M. & Slobedman, B. (2009). Enhanced monocyte Fc phagocytosis by a homologue of interleukin-10 encoded by human cytomegalovirus. *Virology* **391**, 20-24.

Jayawardane, G., Russell, G. C., Thomson, J., Deane, D., Cox, H., Gatherer, D., Ackermann, M., Haig, D. M. & Stewart, J. P. (2008). A captured viral interleukin 10 gene with cellular exon structure. *J Gen Virol* **89**, 2447-2455.

Jenkins, J., Malyak, M. & Arend, W. (1994). The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. *Lymphokine Cytokine Res* **13**, 47.

Jenkins, C., Abendroth, A. & Slobedman, B. (2004). A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J Virol* **78**, 1440-1447.

Jenkins, C., Garcia, W., Abendroth, A. & Slobedman, B. (2008a). Expression of a human cytomegalovirus latency-associated homolog of interleukin-10 during the productive phase of infection. *Virology* **370**, 285-294.

Jenkins, C., Garcia, W., Godwin, M. J., Spencer, J. V., Stern, J. L., Abendroth, A. & Slobedman, B. (2008b). Immunomodulatory properties of a viral homolog of human interleukin-10 expressed by human cytomegalovirus during the latent phase of infection. *J Virol* **82**, 3736-3750.

Jochum, S., Moosmann, A., Lang, S., Hammerschmidt, W. & Zeidler, R. (2012). The EBV immunoevasins vIL-10 and BNLF2a Protect newly infected B cells from immune recognition and elimination. *PLoS Pathog* **8**, e1002704.

Johnson, D. C. & Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* **43**, 1102-1112.

Jones, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16**, 79-95.

Jones, P. C. & Roizman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. *J Virol* **31**, 299-314.

Jones, B. C., Logsdon, N. J., Josephson, K., Cook, J., Barry, P. A. & Walter, M. R. (2002). Crystal structure of human cytomegalovirus IL-10 bound to soluble human IL-10R1. *Proc Natl Acad Sci U S A* **99**, 9404-9409.

Josephson, K., Logsdon, N. J. & Walter, M. R. (2001). Crystal structure of the IL-10/IL-10R1 complex reveals a shared receptor binding site. *Immunity* **15**, 35-46.

K

Kempter, J. & Bergmann, S. M. (2007). Detection of koi herpesvirus (KHV) genome in wild and farmed fish from Northern Poland. *Aquaculture* **272**, S275-S275.

Kempter, J., Kielpinski, M., Panicz, R., Sadowski, J., Mysłowski, B. & Bergmann, S. M. (2012). Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp. *Bull Eur Assoc Fish Pathol* **32**, 212-219.

Kempter, J., Sadowski, J., Schütze, H., Fischer, U., Dauber, M., Fichtner, D., Panicz, R. & Bergmann, S. M. (2009). Koi herpes virus: Do acipenserid restitution programs pose a threat to carp farms in the disease-free zones? *Acta Ichthyologica et Piscatoria* **39**, 119-126.

Keravala, A., Lechman, E. R., Nash, J., Mi, Z. & Robbins, P. D. (2006). Human, viral or mutant human IL-10 expressed after local adenovirus-mediated gene transfer are equally effective in ameliorating disease pathology in a rabbit knee model of antigen-induced arthritis. *Arthritis Res Ther* **8**, R91.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S. & Bergmann, S. M. (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (*Cyprinus carpio*). *Isr J Aquac Bamidgeh* **62**, 28-37.

Kim, K. N., Watanabe, S., Ma, Y., Thornton, S., Giannini, E. H. & Hirsch, R. (2000). Viral IL-10 and soluble TNF receptor act synergistically to inhibit collagen-induced arthritis following adenovirus-mediated gene transfer. *J Immunol* **164**, 1576-1581.

King, A., Lefkowitz, E., Adams, M. & Carstens, E. (2012). Order of presentation of virus taxonomic descriptions. In *Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 23-36. Edited by A. King, E. Lefkowitz, M. Adams & E. Carstens. London: Elsevier.

Kongchum, P., Sandel, E., Lutzky, S., Hallerman, E. M., Hulata, G., David, L. & Palti, Y. (2011). Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (*Cyprinus carpio*). *Aquaculture* **315**, 417-421.

Kotenko, S. V. & Pestka, S. (2001). Viral IL-10 variants. Cytokine Reference, Two-Volume Set (Individual Version): A Compendium of Cytokines and Other Mediators of Host Defense. In Cytokine Reference, pp.1-13. Edited by F. Marc., D. Scott K., H. Toshio., V. Jan ., N. Nicos A.,O. Joost J. Academic Press.

Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V. & Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci U S A* **97**, 1695-1700.

Kotenko, S. V., Krause, C. D., Izotova, L. S., Pollack, B. P., Wu, W. & Pestka, S. (1997). Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J* **16**, 5894-5903.

Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., Haenen, O. L. M., Sunarto, A., Kholidin, E. B. & other authors (2009). Molecular Epidemiology of Koi Herpesvirus. *Fish Pathol* **44**, 59-66.

L

Lateef, Z., Fleming, S., Halliday, G., Faulkner, L., Mercer, A. & Baird, M. (2003). Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. *J Gen Virol* **84**, 1101-1109.

Le Morvan, C., Troutaud, D. & Deschaux, P. (1998). Differential effects of temperature on specific and nonspecific immune defences in fish. *J Exp Biol* **201**, 165-168.

Lechman, E. R., Jaffurs, D., Ghivizzani, S. C., Gambotto, A., Kovesdi, I., Mi, Z., Evans, C. H. & Robbins, P. D. (1999). Direct adenoviral gene transfer of viral IL-10 to rabbit knees with experimental arthritis ameliorates disease in both injected and contralateral control knees. *J Immunol* **163**, 2202-2208.

Lee, M. A., Diamond, M. E. & Yates, J. L. (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**, 2974-2982.

Lee, H. J., Essani, K. & Smith, G. L. (2001). The genome sequence of Yaba-like disease virus, a yatapoxvirus. *Virology* **281**, 170-192.

Lemaire, M. M., Vanhaudenarde, A., Nizet, Y., Dumoutier, L. & Renauld, J. C. (2011). Induction of autoantibodies against mouse soluble proteins after immunization with living cells presenting the autoantigen at the cell surface in fusion with a human type 2 transmembrane protein. *J Immunol Methods* **367**, 56-62.

Lin, Y. L., Chang, P. C., Wang, Y. & Li, M. (2008). Identification of novel viral interleukin-10 isoforms of human cytomegalovirus AD169. *Virus Res* **131**, 213-223.

Liu, Y., Wei, S. H., Ho, A. S., de Waal Malefyt, R. & Moore, K. W. (1994). Expression cloning and characterization of a human IL-10 receptor. *J Immunol* **152**, 1821-1829.

Liu, Y., de Waal Malefyt, R., Briere, F., Parham, C., Bridon, J. M., Banchereau, J., Moore, K. W. & Xu, J. (1997). The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor. *J Immunol* **158**, 604-613.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402-408.

Lockridge, K. M., Zhou, S. S., Kravitz, R. H., Johnson, J. L., Sawai, E. T., Blewett, E. L. & Barry, P. A. (2000). Primate cytomegaloviruses encode and express an IL-10-like protein. *Virology* **268**, 272-280.

Logsdon, N. J., Eberhardt, M. K., Allen, C. E., Barry, P. A. & Walter, M. R. (2011). Design and analysis of rhesus cytomegalovirus IL-10 mutants as a model for novel vaccines against human cytomegalovirus. *PloS one* **6**, e28127.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M. & Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* **82**, 10436-10443.

M

Ma, Y., Thornton, S., Duwel, L. E., Boivin, G. P., Giannini, E. H., Leiden, J. M., Bluestone, J. A. & Hirsch, R. (1998). Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. *J Immunol* **161**, 1516-1524.

Magel, G. D. & Tyring, S. (2012). *Herpesviridae- a look into this unique family of viruses*. Shanghai: in Tech.

Markine-Goriaynoff, N., Gillet, L., Karlsen, O. A., Haarr, L., Minner, F., Pastoret, P.-P., Fukuda, M. & Vanderplasschen, A. (2004). The core 2 β-1, 6-N-acetylglucosaminyltransferase-M encoded by bovine herpesvirus 4 is not essential for virus replication despite contributing to post-translational modifications of structural proteins. *J Gen Virol* **85**, 355-367.

Masse, M. J., Jons, A., Dijkstra, J. M., Mettenleiter, T. C. & Flamand, A. (1999). Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. *J Virol* **73**, 10503-10507.

Mastroleo, F., Leroy, B., Van Houdt, R., s' Heeren, C., Mergeay, M., Hendrickx, L. & Wattiez, R. (2009). Shotgun proteome analysis of Rhodospirillum rubrum S1H: integrating data from gel-free and gel-based peptides fractionation methods. *J Proteome Res* **8**, 2530-2541.

Matsuda, M., Salazar, F., Petersson, M., Masucci, G., Hansson, J., Pisa, P., Zhang, Q. J., Masucci, M. G. & Kiessling, R. (1994). Interleukin 10 pretreatment protects target cells from tumor- and allo-specific cytotoxic T cells and downregulates HLA class I expression. *J Exp Med* **180**, 2371-2376.

McGeoch, D. J., Rixon, F. J. & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Res* **117**, 90-104.

McGeoch, D. J., Davison, A. J., Dolan, A., Gatherer, D. & Sevilla-Reyes, E. E. (2008). Molecular evolution of the Herpesvirales. In *Origin and evolution of viruses*, 2 edn, pp. 447-476. Edited by E. Domingo, C. R. Parrish & J. J. Holland. Amsterdam: Elsevier.

Meier-Trummer, C. S., Tobler, K., Hilbe, M., Stewart, J. P., Hart, J., Campbell, I., Haig, D. M., Glauser, D. L., Ehrensperger, F. & other authors (2009). Ovine herpesvirus 2 structural proteins in epithelial cells and M-cells of the appendix in rabbits with malignant catarrhal fever. *Vet Microbiol* **137**, 235-242.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-180.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

Michel, B., Fournier, G., Lieffrig, F., Costes, B. & Vanderplasschen, A. (2010). Cyprinid herpesvirus 3. *Emerg Infect Dis* **16**, 1835-1843.

Michel, B., Leroy, B., Stalin Raj, V., Lieffrig, F., Mast, J., Wattiez, R., Vanderplasschen, A. F. & Costes, B. (2010). The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *J Gen Virol* **91**, 452-462.

Minamoto, T., Honjo, M. N., Yamanaka, H., Uchii, K. & Kawabata, Z. (2012). Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan. *Res Vet Sci* **93**, 508-514.

Minamoto, T., Honjo, M. N., Yamanaka, H., Tanaka, N., Itayama, T. & Kawabata, Z. (2011). Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Res Vet Sci* **90**, 530-532.

Minamoto, T., Honjo, M. N., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2009). Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Vet Microbiol* **135**, 261-266.

Miwa, S., Ito, T. & Sano, M. (2007). Morphogenesis of koi herpesvirus observed by electron microscopy. *J Fish Dis* **30**, 715-722.

Miyazaki, I., Cheung, R. K. & Dosch, H. M. (1993). Viral interleukin 10 is critical for the induction of B cell growth transformation by Epstein-Barr virus. *J Exp Med* **178**, 439-447.

Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M. & Kobayashi, T. (2008). Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Dis Aquat Organ* **80**, 1-11.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683-765.

Moore, K. W., O'garra, A., Malefyt, R. W., Vieira, P. & Mosmann, T. R. (1993). Interleukin-10. *Annu Rev Immunol* **11**, 165-190.

Moore, K. W., Vieira, P., Fiorentino, D. F., Trounstine, M. L., Khan, T. A. & Mosmann, T. R. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science* **248**, 1230-1234.

Mosser, D. M. & Zhang, X. (2008). Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* **226**, 205-218.

N

Nachtwey, J. & Spencer, J. V. (2008). HCMV IL-10 suppresses cytokine expression in monocytes through inhibition of nuclear factor-κ B. *Viral Immunol* **21**, 477-482.

Nast, C. C., Moudgil, A., Zuo, X. J., Toyoda, M. & Jordan, S. C. (1997). Long-term allograft acceptance in a patient with posttransplant lymphoproliferative disorder: correlation with intragraft viral interleukin-10. *Transplantation* **64**, 1578-1582.

Neukirch, M. & Kunz, U. (2001). Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality. *Bull Eur Assoc Fish Pathol* **21**, 125-135.

Neukirch, M., Böttcher, K. & Bunnajirakul, S. (1999). Isolation of a virus from koi with altered gills. *Bull Eur Assoc Fish Pathol* **19**, 221-224.

Niiro, H., Otsuka, T., Abe, M., Satoh, H., Ogo, T., Nakano, T., Furukawa, Y. & Niho, Y. (1992). Epstein-Barr virus BCRF1 gene product (viral interleukin 10) inhibits superoxide anion production by human monocytes. *Lymphokine Cytokine Res* **11**, 209-214.

Niiro, H., Otsuka, T., Kuga, S., Nemoto, Y., Abe, M., Hara, N., Nakano, T., Ogo, T. & Niho, Y. (1994). IL-10 inhibits prostaglandin E2 production by lipopolysaccharide-stimulated monocytes. *Int Immunol* **6**, 661-664.

O

Odom, M. R., Hendrickson, R. C. & Lefkowitz, E. J. (2009). Poxvirus protein evolution: family wide assessment of possible horizontal gene transfer events. *Virus Res* **144**, 233-249.

Oh, M. J., Jung, S.J., Choi, T.J., Kim, H.R., Rajendran, K.V., Kim, Y.J., Park, M.A., Chun, S.K. (2001). A viral disease occurring in cultured carp Cyprinus carpio in Korea. *Fish Pathol* **36**, 147-151.

OIE (World Organisation for Animal Health) (2012): *Report of the meeting of the OIE aquatic animal health standards commission.* Paris. http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Aquatic_Commission/A_AAC_March_2012.pdf.

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A. & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* **29**, 71-109.

Ouyang, P., Rakus, K., Boutier, M., Reschner, A., Leroy, B., Ronsmans, M., Fournier, G., Scohy, S., Costes, B. & other authors (2013). The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. *Vet Res* **44**, 53.

P

Pepperl-Klindworth, S., Besold, K., Frankenberg, N., Farkas, M., Kuball, J., Theobald, M. & Plachter, B. (2006). Cytomegalovirus interleukin-10 expression in infected cells does not impair MHC class I restricted peptide presentation on bystanding antigen-presenting cells. *Viral Immunol* **19**, 92-101.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured *Cyprinus Carpio* in Israel. *Isr J Aquac Bamidgeh* **55**, 5-12.

Perelberg, A., Ilouze, M., Kotler, M. & Steinitz, M. (2008). Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). *Vaccine* **26**, 3750-3756.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y. & Kotler, M. (2005). Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. *Vaccine* **23**, 3396-3403.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. & Fisher, P. B. (2004). Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* **22**, 929-979.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., van Dyk, L. F., Ho, C. K., Shuman, S. & other authors (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods* **2**, 269-276.

Piackova, V., Flajshans, M., Pokorova, D., Reschova, S., Gela, D., Cizek, A. & Vesely, T. (2013). Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV). *J Fish Dis* **36**, 75-80.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D. & other authors (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J Virol* **78**, 9544-9551.

Pinto, R. D., Nascimento, D. S., Reis, M. I., do Vale, A. & Dos Santos, N. M. (2007). Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax L.*) interleukin-10. *Mol Immunol* **44**, 2056-2065.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G. & other authors (2012). The Pfam protein families database. *Nucleic Acids Res* **40**, D290-301.

Q

Qin, L., Chavin, K. D., Ding, Y., Tahara, H., Favaro, J. P., Woodward, J. E., Suzuki, T., Robbins, P. D., Lotze, M. T. & other authors (1996). Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J Immunol* **156**, 2316-2323.

R

Radosavljević, V., Jeremić, S., Ćirković, M., Lako, B., Milićević, V., Potkonjak, A. & Nikolin, V. (2012). Common fish species in polyculture with carp as cyprinid herpes virus 3 carriers. *Acta Veterinaria* **62**, 675-681.

Raftery, M. J., Wieland, D., Gronewald, S., Kraus, A. A., Giese, T. & Schönrich, G. (2004). Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. *J Immunol* **173**, 3383-3391.

Raftery, M. J., Hitzler, M., Winau, F., Giese, T., Plachter, B., Kaufmann, S. H. E. & Schönrich, G. (2008). Inhibition of CD1 antigen presentation by human cytomegalovirus. *J Virol* **82**, 4308-4319.

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., Delforges, C., Costes, B., Farnir, F. & other authors (2011). Skin mucus of Cyprinus carpio inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Vet Res* **42**, 92.

Rakus, K. L., Wiegertjes, G. F., Adamek, M., Siwicki, A. K., Lepa, A. & Irnazarow, I. (2009). Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II B gene polymorphism. *Fish Shellfish Immunol* **26**, 737-743.

Rakus, K. L., Irnazarow, I., Adamek, M., Palmeira, L., Kawana, Y., Hirono, I., Kondo, H., Matras, M., Steinhagen, D. & other authors (2012). Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. *Dev Comp Immunol* **37**, 65-76.

Roizman, B. & Pellet, P. E. (2007). The Family: Herpesviridae a brief introduction. In *Fields virology*, 5 edn, pp. 2479-2499. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams and Wilkins.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. & Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**, 425-449.

Romalde, J. L., Luzardo-Alvárez, A., Ravelo, C., Toranzo, A. E. & Blanco-Méndez, J. (2004). Oral immunization using alginate microparticles as a useful strategy for booster vaccination against fish lactoccocosis. *Aquaculture* **236**, 119-129.

Rombout, J., Huttenhuis, H., Picchietti, S. & Scapigliati, G. (2005). Phylogeny and ontogeny of fish leucocytes. *Fish Shellfish Immunol* **19**, 441-455.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M. & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* **21**, 4677-4684.

Ronsmans M, Rougeot C, Melard C & Vanderplasschen A (2012). Susceptibility of *Cyprinus carpio* to Cyprinid herpesvirus-3 at the early stages of life. *The Netherlands: Fish Immunology Workshop, Wageningen*.

Rosenkranz, D., Klupp, B. G., Teifke, J. P., Granzow, H., Fichtner, D., Mettenleiter, T. C. & Fuchs, W. (2008). Identification of envelope protein pORF81 of koi herpesvirus. *J Gen Virol* **89**, 896-900.

Rouas-Freiss, N., Gonçalves, R. M. B., Menier, C., Dausset, J. & Carosella, E. D. (1997). Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci U S A* **94**, 11520-11525.

Rousset, F., Garcia, E., Defrance, T., Péronne, C., Vezzio, N., Hsu, D. H., Kastelein, R., Moore, K. W. & Banchereau, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* **89**, 1890-1893.

Rowbottom, A., Lepper, M., Garland, R., Cox, C., Corley, E., Oakhill, A. & Steward, C. (1999). Interleukin-10-induced CD8 cell proliferation. *Immunology* **98**, 80-89.

S

Sabat, R. (2010). IL-10 family of cytokines. *Cytokine Growth Factor Rev* **21**, 315-324.

Sabat, R., Grutz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K. & Geginat, J. (2010). Biology of interleukin-10. *Cytokine Growth Factor Rev* **21**, 331-344.

Salek-Ardakani, S., Arrand, J. R. & Mackett, M. (2002a). Epstein-Barr virus encoded interleukin-10 inhibits HLA-class I, ICAM-1, and B7 expression on human monocytes: implications for immune evasion by EBV. *Virology* **304**, 342-351.

Salek-Ardakani, S., Stuart, A. D., Arrand, J. E., Lyons, S., Arrand, J. R. & Mackett, M. (2002b). High level expression and purification of the Epstein-Barr virus encoded cytokine viral interleukin 10: efficient removal of endotoxin. *Cytokine* **17**, 1-13.

Santin, A. D., Hermonat, P. L., Ravaggi, A., Bellone, S., Pecorelli, S., Roman, J. J., Parham, G. P. & Cannon, M. J. (2000). Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8+ cytotoxic T lymphocytes. *J Virol* **74**, 4729-4737.

Savan, R., Igawa, D. & Sakai, M. (2003). Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. *Eur J Biochem* **270**, 4647-4654.

Savin, K. W., Cocks, B. G., Wong, F., Sawbridge, T., Cogan, N., Savage, D. & Warner, S. (2010). A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J* **7**, 308.

Shackelton, L. A. & Holmes, E. C. (2004). The evolution of large DNA viruses: combining genomic information of viruses and their hosts. *Trends Microbiol* **12**, 458-465.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G. & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. *Aquaculture* **245**, 1-11.

Shimizu, T., Yoshida, N., Kasai, H. & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathol* **41**, 153-157.

Siwicki, A., Kazuń, K., Kazuń, B. & Majewicz-Zbikowska, E. (2012). Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes. *Arch Pol Fish* **20**, 123-128.

Slobedman, B., Barry, P. A., Spencer, J. V., Avdic, S. & Abendroth, A. (2009). Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. *J Virol* **83**, 9618-9629.

Smith, J. D. (1980). An additional role for the outer nuclear membrane in the morphogenesis of herpes simplex virus. *Intervirology* **13**, 312-316.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007-1021.

Sommerset, I., Krossøy, B., Biering, E. & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Rev Vaccines* **4**, 89-101.

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-410.

Spencer, J. V. (2007). The cytomegalovirus homolog of interleukin-10 requires phosphatidylinositol 3-kinase activity for inhibition of cytokine synthesis in monocytes. *J Virol* **81**, 2083-2086.

Spencer, J. V., Cadaoas, J., Castillo, P. R., Saini, V. & Slobedman, B. (2008). Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10. *Virology* **374**, 164-169.

Spencer, J. V., Lockridge, K. M., Barry, P. A., Lin, G., Tsang, M., Penfold, M. E. T. & Schall, T. J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J Virol* **76**, 1285-1292.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P. & Way, K. (2009). Antibody response of two populations of common carp, *Cyprinus carpio* L., exposed to koi herpesvirus. *J Fish Dis* **32**, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P. & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Dis Aquat Organ* **67**, 15-23.

Stuart, A. D., Stewart, J. P., Arrand, J. R. & Mackett, M. (1995). The Epstein-Barr virus encoded cytokine viral interleukin-10 enhances transformation of human B lymphocytes. *Oncogene* **11**, 1711-1720.

Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C. & Walker, P. J. (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. *J Fish Dis* **34**, 87-101.

Sunarto, A., Liongue, C., McColl, K. A., Adams, M. M., Bulach, D., Crane, M. S. J., Schat, K. A., Slobedman, B., Barnes, A. C. & other authors (2012). Koi herpesvirus encodes and expresses a functional interleukin-10. *J Virol* **86**, 11512-11520.

Syakuri, H., Adamek, M., Brogden, G., Rakus, K. L., Matras, M., Irnazarow, I. & Steinhagen, D. (2013). Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: Molecular identification and regulation of the mRNA expression of claudin encoding genes. *Fish Shellfish Immunol* **34**, 305-314.

T

Takanaski, S., Nonaka, R., Xing, Z., O'Byrne, P., Dolovich, J. & Jordana, M. (1994). Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med* **180**, 711-715.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-2739.

Telford, E. A. R., Watson, M. S., Aird, H. C., Perry, J. & Davison, A. J. (1995). The DNA sequence of equine herpesvirus 2. *J Mol Biol* **249**, 520-528.

Terhune J, S., Grizzle J, M., Hayden K, McClenahan S, D., Lamprecht S, D. & White M, G. (2004). First report of koi herpesvirus in wild common carp in the western hemisphere. *Fish Health Newsletter* **32**, 8-9.

Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W. & Rennick, D. M. (1991). Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *J Exp Med* **173**, 507-510.

Tomé, A. R., Kuś, K., Correia, S., Paulo, L. M., Zacarias, S., de Rosa, M., Figueiredo, D., Parkhouse, R. M. & Athanasiadis, A. (2013). Crystal structure of a poxvirus-like zalpha domain from cyprinid herpesvirus 3. *J Virol* **87**, 3998-4004.

Touitou, R., Cochet, C. & Joab, I. (1996). Transcriptional analysis of the Epstein-Barr virus interleukin-10 homologue during the lytic cycle. *J Gen Virol* **77 (Pt 6)**, 1163-1168.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Kutish, G. & Rock, D. (2001). Genome of lumpy skin disease virus. *J Virol* **75**, 7122-7130.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Kutish, G. & Rock, D. (2004). The genome of canarypox virus. *J Virol* **78**, 353-366.

Tulman, E., Afonso, C., Lu, Z., Zsak, L., Sur, J. H., Sandybaev, N., Kerembekova, U., Zaitsev, V., Kutish, G. & other authors (2002). The genomes of sheeppox and goatpox viruses. *J Virol* **76**, 6054-6061.

U

Uchii, K., Matsui, K., Iida, T. & Kawabata, Z. (2009). Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, *Cyprinus carpio* L. *J Fish Dis* **32**, 857-864.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780-783.

V

van Beurden, S. J., Peeters, B. P., Rottier, P. J., Davison, A. J. & Engelsma, M. Y. (2013). Genome-wide gene expression analysis of anguillid herpesvirus 1. *BMC Genomics* **14**, 83.

van Beurden, S. J., Forlenza, M., Westphal, A. H., Wiegertjes, G. F., Haenen, O. L. & Engelsma, M. Y. (2011). The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish Shellfish Immunol* **31**, 1211-1217.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

van Beurden, S. J., Engelsma, M. Y., Roozenburg, I., Voorbergen-Laarman, M. A., van Tulden, P. W., Kerkhoff, S., van Nieuwstadt, A. P., Davidse, A. & Haenen, O. (2012). Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands. *Dis Aquat Organ* **101**, 69-86.

van Putten, S., Wübben, M., Hennink, W., Van Luyn, M. & Harmsen, M. (2009). The downmodulation of the foreign body reaction by cytomegalovirus encoded interleukin-10. *Biomaterials* **30**, 730-735.

Vieira, P., de Waal-Malefyt, R., Dang, M., Johnson, K., Kastelein, R., Fiorentino, D., Roncarolo, M., Mosmann, T. & Moore, K. (1991). Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc Natl Acad Sci U S A* **88**, 1172-1176.

Vrancken, R., Boutier, M., Ronsmans, M., Reschner, A., Leclipteux, T., Lieffrig, F., Collard, A., Mélard, C., Wera, S. & other authors (2013). Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs. *J Virol Methods* **accepted**.

W

Wagner, E. K., Petroski, M. D., Pande, N. T., Lieu, P. T. & Rice, M. (1998). Analysis of factors influencing kinetics of herpes simplex virus transcription utilizing recombinant virus. *Methods* **16**, 105-116.

Walter, M. R. & Nagabhushan, T. L. (1995). Crystal structure of interleukin 10 reveals an interferon gamma-like fold. *Biochemistry* **34**, 12118-12125.

Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J. & Hedrick, R. P. (2009). Phylogenetic relationships in the family Alloherpesviridae. *Dis Aquat Organ* **84**, 179-194.

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., Hirono, I., Aoki, T., Davison, A. J. & other authors (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*. *J Gen Virol* **86**, 1659-1667.

Wang, F. Z., Weber, F., Croce, C., Liu, C. G., Liao, X. & Pellett, P. E. (2008). Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *J Virol* **82**, 9065-9074.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. & Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using *gal*K selection. *Nucleic Acids Res* **33**, e36.

Whalen, J. D., Lechman, E. L., Carlos, C. A., Weiss, K., Glorioso, J. C., Robbins, P. D. & Evans, C. H. (1999). Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J Immunol* **162**, 3625-3632.

Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepfe, E., Ackermann, M., Mueller, M. & other authors (2005). Impairment of nuclear pores in bovine herpesvirus 1-infected MDBK cells. *J Virol* **79**, 1071-1083.

Willems, F., Marchant, A., Delville, J. P., Gérard, C., Delvaux, A., Velu, T., De Boer, M. & Goldman, M. (1994). Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur J Immunol* **24**, 1007-1009.

Wise, L., McCaughan, C., Tan, C. K., Mercer, A. A. & Fleming, S. B. (2007). Orf virus interleukin-10 inhibits cytokine synthesis in activated human THP-1 monocytes, but only partially impairs their proliferation. *J Gen Virol* **88**, 1677-1682.

Wolf, K. & Dalrlingtron, R. W. (1971). Channel catfish virus: a new herpesvirus of ictalurid fish. *J Virol* **8**, 525-533.

Wolk, K., Witte, E., Reineke, U., Witte, K., Friedrich, M., Sterry, W., Asadullah, K., Volk, H. & Sabat, R. (2004). Is there an interaction between interleukin-10 and interleukin-22? *Genes Immun* **6**, 8-18.

X

Xu, J., Zeng, L., Zhang, H., Zhou, Y., Ma, J. & Fan, Y. (2013). Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, *Carassius auratus gibelio* in China. *Vet Microbiol* **166**, 138-144.

Xu, Z., Iwatsuki, K., Oyama, N., Ohtsuka, M., Satoh, M., Kikuchi, S., Akiba, H. & Kaneko, F. (2001). The latency pattern of Epstein–Barr virus infection and viral IL-10 expression in cutaneous natural killer/T-cell lymphomas. *Br J Cancer* **84**, 920.

Xu, J. R., Bently, J., Beck, L., Reed, A., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Rockey, D. D. & Jin, L. (2013). Analysis of koi herpesvirus latency in wild common carp and ornamental koi in Oregon, USA. *J Virol Methods* **187**, 372-379.

Y

Yamamoto-Tabata, T., McDonagh, S., Chang, H. T., Fisher, S. & Pereira, L. (2004). Human cytomegalovirus interleukin-10 downregulates metalloproteinase activity and impairs endothelial cell migration and placental cytotrophoblast invasiveness in vitro. *J Virol* **78**, 2831-2840.

Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T. & Miyazaki, T. (2006). Oral immunization of common carp with a liposome vaccine fusing koi herpesvirus antigen. *Fish pathology* **41**, 141-145.

Yoon, S. I., Jones, B. C., Logsdon, N. J. & Walter, M. R. (2005). Same structure, different function crystal structure of the Epstein-Barr virus IL-10 bound to the soluble IL-10R1 chain. *Structure* **13**, 551-564.

Yoon, S. I., Logsdon, N. J., Sheikh, F., Donnelly, R. P. & Walter, M. R. (2006). Conformational changes mediate interleukin-10 receptor 2 (IL-10R2) binding to IL-10 and assembly of the signaling complex. *J Biol Chem* **281**, 35088-35096.

Yu, X., Cheng, Y., Shi, B., Qian, F., Wang, F., Liu, X., Yang, H., Xu, Q., Qi, T. & other authors (2008). Measles virus infection in adults induces production of IL-10 and is associated with increased $CD4^+$ $CD25^+$ regulatory T cells. *J Immunol* **181**, 7356-7366.

Yuasa, K. & Sano, M. (2009). Koi herpesvirus: Status of outbreaks, diagnosis, surveillance, and research. *Isr J Aquac Bamidgeh* **61**, 169-179.

Yuasa, K., Ito, T. & Sano, M. (2008). Effect of water temperature on mortality and virus shedding in carp experimentally infected with Koi herpesvirus. *Fish Pathol* **43**, 83-85.

Yuasa, K., Kurita, J., Kawana, M., Kiryu, I., Oseko, N. & Sano, M. (2012). Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. *Dis Aquat Organ* **100**, 11-18.

Yue, Y. & Barry, P. A. (2008). Rhesus cytomegalovirus: a nonhuman primate model for the study of human cytomegalovirus. *Adv Virus Res* **72**, 207-226.

Z

Zdanov, A. (2004). Structural features of the interleukin-10 family of cytokines. *Curr Pharm Des* **10**, 3873-3884.

Zdanov, A., Schalk-Hihi, C. & Wlodawer, A. (1996). Crystal structure of human interleukin-10 at 1.6 Å resolution and a model of a complex with its soluble receptor. *Protein Sci* **5**, 1955-1962.

Zdanov, A., Schalk-Hihi, C., Menon, S., Moore, K. W. & Wlodawer, A. (1997). Crystal structure of Epstein-Barr virus protein BCRF1, a homolog of cellular interleukin-10. *J Mol Biol* **268**, 460-467.

Zdanov, A., Schalk-Hihi, C., Gustchina, A., Tsang, M., Weatherbee, J. & Wlodawer, A. (1995). Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. *Structure* **3**, 591-601.

Zhang, D. C., Shao, Y. Q., Huang, Y. Q. & Jiang, S. G. (2005). Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerion*). *J Biochem Mol Biol* **38**, 571-576.

Presses de la Faculté de Médecine Vétérinaire de l'Université de Liège

4000 Liège (Belgique)

D/2013/0480/16
ISBN 978-2-87543-038-0