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.
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Promotor : Prof. Alain Vanderplasschen

Ping OUYANG

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« 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.
Acknowledgments

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 Léta, 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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D-LC MS/MS:</td>
<td>Two-dimensional liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>α gene:</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>aa:</td>
<td>Amino acid</td>
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<tr>
<td>ACHV:</td>
<td>Atlantic cod herpesvirus</td>
</tr>
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<td>AciHV-1:</td>
<td>Acipenserid herpesvirus 1</td>
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</tr>
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<tr>
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</tr>
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<td>APCs:</td>
<td>Antigen presenting cells</td>
</tr>
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<td>AtHV-3:</td>
<td>Ateline herpesvirus 3</td>
</tr>
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<td>Au:</td>
<td>Goldfish fin cell</td>
</tr>
<tr>
<td>β gene :</td>
<td>Early gene</td>
</tr>
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<td>BAC:</td>
<td>Bacterial artificial chromosome</td>
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</tr>
<tr>
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<td>Baboon lymphocryptovirus</td>
</tr>
<tr>
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<td>Bovin herpesvirus 4</td>
</tr>
<tr>
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<td>Bone marrow-derived dendritic cells</td>
</tr>
<tr>
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<td>Bovine herpesvirus 1</td>
</tr>
<tr>
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<td>Bovine herpesvirus 4</td>
</tr>
<tr>
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<td>Bovine herpesvirus 5</td>
</tr>
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<td>Bonobo herpesvirus</td>
</tr>
<tr>
<td>bp:</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPSV:</td>
<td>Bovine papular stomatitis virus</td>
</tr>
<tr>
<td>CaF-2:</td>
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</tr>
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<td>CCB:</td>
<td><em>Cyprinus carpio</em> brain cell</td>
</tr>
<tr>
<td>CCG:</td>
<td><em>Cyprinus carpio</em> gill cell</td>
</tr>
<tr>
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<td>Chimpanzee cytomegalovirus</td>
</tr>
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<td>CCV:</td>
<td>Channel catfish virus (= IcHV-1)</td>
</tr>
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<td>CD:</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA:</td>
<td>Complementary DNA</td>
</tr>
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<td>Cercopithecine herpesvirus 2</td>
</tr>
<tr>
<td>CeHV-9:</td>
<td>Cercopithecine herpesvirus 9</td>
</tr>
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<td>Carp herpesvirus (= CyHV-1)</td>
</tr>
<tr>
<td>CHX:</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>cIL-10:</td>
<td>Cellular Interleukin-10</td>
</tr>
<tr>
<td>CMV:</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNGV:</td>
<td>Carp nephritis and gill necrosis virus</td>
</tr>
<tr>
<td>CNPV:</td>
<td>Canarypox virus</td>
</tr>
<tr>
<td>CPE:</td>
<td>Cytopathic effect</td>
</tr>
</tbody>
</table>
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)
galK: 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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-4</td>
<td>Human herpesvirus 4</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>IcmHV</td>
<td>Ictalurus melas herpesvirus (= IcHV-1)</td>
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<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IgA, G, M</td>
<td>Immunoglobulin A, G, M</td>
</tr>
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<td>IL</td>
<td>Interleukin</td>
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<td>IL-10R</td>
<td>IL-10 receptor</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IPNV</td>
<td>Infectious pancreatic necrosis virus</td>
</tr>
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<td>IR</td>
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<td>IVIS</td>
<td><em>in vivo</em> bioluminescence imaging system</td>
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<td>Late</td>
</tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>Luciferase</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>MEM</td>
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<td>MHC class II B</td>
<td>Major Histocompatibility Complex class II B</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
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<td>Abbreviation</td>
<td>Official Name</td>
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<tr>
<td>--------------</td>
<td>-------------------------------------</td>
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<td>mRNA</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>NGF-2</td>
<td>Epithelial-like cell line from fins of coloured carp 2</td>
</tr>
<tr>
<td>NGF-3</td>
<td>Epithelial-like cell line from fins of coloured carp 3</td>
</tr>
<tr>
<td>NK cells</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td>Open reading frame</td>
</tr>
<tr>
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</tr>
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<td>Ostreid herpesvirus 1</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>p.f.u.</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
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<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<td>Pseudorabies virus</td>
</tr>
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</tr>
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<td>RELP</td>
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<td>RT-PCR</td>
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<td>RTR</td>
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</tr>
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<tr>
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<td>Salmonid herpesvirus 3</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SHV</td>
<td>Steelhead herpesvirus</td>
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<td>sIL10 R1</td>
<td>Soluble IL10 receptor 1</td>
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<td>Abbreviation</td>
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<td>SMCMV:</td>
<td>Squirrel monkey cytomegalovirus</td>
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<td>Sheeppox virus</td>
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<td>T helper 1</td>
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<td>Tumor necrosis factor receptor</td>
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</tr>
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<td>Viral IL-10</td>
</tr>
<tr>
<td>VNTR:</td>
<td>Variable number of tandem repeats</td>
</tr>
</tbody>
</table>
Preamble .............................................................................................................................................. 1

Introduction ........................................................................................................................................... 3

1st chapter:

“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:

“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
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
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st chapter:</td>
<td>75</td>
</tr>
<tr>
<td>“The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication <em>in vitro</em> nor for virulence <em>in vivo</em>”</td>
<td></td>
</tr>
<tr>
<td>2nd chapter:</td>
<td>95</td>
</tr>
<tr>
<td>“Development of a Safe and Efficacious Attenuated Recombinant Vaccine against Cyprinid herpesvirus 3”</td>
<td></td>
</tr>
<tr>
<td>Discussion and perspectives</td>
<td>112</td>
</tr>
<tr>
<td>Summary</td>
<td>119</td>
</tr>
<tr>
<td>References</td>
<td>122</td>
</tr>
</tbody>
</table>
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 carp 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 describe 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*b and *ca* are inversed and are separated by long (UL) and short (US) unique sequences. In the B group, terminal sequences are repeated a variable number of times at each extremity. In the D group, US can be inverted compared to the UL giving two different isomers. In the E group, UL and US 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).

<table>
<thead>
<tr>
<th></th>
<th>A LTR RTR</th>
<th>B R1 R2 R3 R4</th>
<th>C R1 R2 R3 R4</th>
<th>D UL IR US ER</th>
<th>E UL UL US US ER ER ER</th>
<th>F</th>
<th>Isomers</th>
<th>Examples</th>
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<th>Isomers</th>
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<td>1</td>
<td>HHV-6 CyHV-3</td>
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<td>AlHV-1 BoHV-4</td>
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<td>1</td>
<td>HHV-4</td>
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<tr>
<td>2</td>
<td>BoHV-1</td>
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<td>4</td>
<td>HHV-1</td>
</tr>
<tr>
<td>1</td>
<td>MuHV-1</td>
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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.,
Attachment of the virion on the cell surface and fusion of the envelope with the plasma membrane

Viral protein expression and viral DNA replication

Immediate Early

Early

Late

Viral egress

Cytoplasm

Nucleus

Concatemers

Luminal model

Enveloppement-deenveloppement model

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-de-envelopment 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).
Introduction: The order *Herpesvirales*

(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 through 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)
Introduction: The order Herpesvirales

(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 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 Abulone Herpesvirus (AbHV) and OsHV-1, it has been proposed to
### Table 1. Herpesviruses of fish and amphibians (adapted from Hallon et al. 2011).

<table>
<thead>
<tr>
<th>Virus name (abbreviation)</th>
<th>Clade</th>
<th>Common name (abbreviation)</th>
<th>Host(s)</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anguillid HV 1 (AngHV-1)</td>
<td>1</td>
<td>HV anguillae (HVA)</td>
<td>Japanese eel Anguilla japonica and European eel A. Anguilla</td>
<td>Haemorrhages of skin, fins, gills, liver</td>
</tr>
<tr>
<td>Cyprinid HV 1 (CyHV-1)</td>
<td>1</td>
<td>HV cyprini, carp pox HV, carp HV(CHV)</td>
<td>Common carp Cyprinus carpio</td>
<td>High losses in fry- exophthalmia haemorrhages, survivors have papilloma</td>
</tr>
<tr>
<td>Cyprinid HV 2 (CyHV-2)</td>
<td>1</td>
<td>Goldfish hematopoietic necrosis virus (GHNV)</td>
<td>Goldfish Carassius auratus</td>
<td>High mortality at all ages. Necrosis of hematopoietic tissue, spleen, pancreas, intestine</td>
</tr>
<tr>
<td>Cyprinid HV 3 (CyHV-3)</td>
<td>1</td>
<td>Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)</td>
<td>Common carp</td>
<td>Gill inflammation, hyperplasia, and necrosis, hematopoietic tissue necrosis. High mortality at all ages</td>
</tr>
<tr>
<td>Ictalurid HV 1 (IcHV-1)</td>
<td>2</td>
<td>Channel catfish virus (CCV), Channel catfish herpesvirus</td>
<td>Channel catfish Ictalurus punctatus</td>
<td>Kidney, liver and intestinal necrosis, haemorrhages, high mortality in young subjects</td>
</tr>
<tr>
<td>Ictalurid HV 2 (IcHV-2)</td>
<td>2</td>
<td>Ictalurus melas HV (kmcHV)</td>
<td>Black bullhead Amia melas</td>
<td>Kidney necrosis, haemorrhages, high mortality at all ages</td>
</tr>
<tr>
<td>Acipenserid HV 1 (AcHV-1)</td>
<td>2</td>
<td>White sturgeon HV 1</td>
<td>White sturgeon Acipenser transmontanus</td>
<td>diffuse dermatitis, high losses in juveniles</td>
</tr>
<tr>
<td>Sal monid HV 2 (SalHV-2)</td>
<td>2</td>
<td>Onchorhyncus masou virus (OMV)</td>
<td>Cherry salmon O. masou, coho salmon O. kisutch, sockeye salmon O. nerka, coho salmon O. keta, rainbow trout,</td>
<td>Viremia, external haemorrhages exophthalmia, hepatic necrosis. High mortality in young subjects. Survivors have oral papilloma. Infected female shed virus in ovarian fluid</td>
</tr>
<tr>
<td>Salmonid HV 3 (SalHV-3)</td>
<td>2</td>
<td>Epizootic epitheliotropic disease virus (EEDV)</td>
<td>Lake trout Salvelinus namaycush, lake trout × brook trout S. fontinalis hybrids</td>
<td>Epithelial hyperplasia, hypertrophy, haemorrhages on eye and jaw. High mortality in juveniles at 6–15 °C</td>
</tr>
<tr>
<td>Ranid HV 1 (RaHV-1)</td>
<td>2</td>
<td>Lucké tumor HV (LTHV)</td>
<td>Leopard frog Rana pipiens</td>
<td>Renal adenocarcinoma</td>
</tr>
<tr>
<td>Ranid HV 2 (RaHV-2)</td>
<td>2</td>
<td>Frog virus 4 (FV-4)</td>
<td>Leopard frog</td>
<td>No known disease</td>
</tr>
<tr>
<td>Percid HV 1 (PeHV-1)</td>
<td>2</td>
<td>TIlapia larvae encephalitis virus (TLEV)</td>
<td>Blue tilapia Oreochromis aureus</td>
<td>Gill inflammation associated with epithelial hyperplasia and hypertrophy. High mortality</td>
</tr>
<tr>
<td>Tilapia HV</td>
<td></td>
<td>Possible Herpesviridae</td>
<td>Blue tilapia Oreochromis aureus</td>
<td>Encephalitis in larvae. High mortality</td>
</tr>
<tr>
<td>Percid HV 1 (PeHV-1)</td>
<td>2</td>
<td>HV vitreum, Walleye HV</td>
<td>Walleye Stizostedion vitreum</td>
<td>Diffuse epidermal hyperplasia</td>
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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 based 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


Introduction: Cyprinid herpesvirus 3

2nd chapter:

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

_Veterinary Research_ (2013), 44:85

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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. AIHV-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: ostrid 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 Cypriniviruses (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

<table>
<thead>
<tr>
<th>Common name (species)</th>
<th>Detection of CyHV-3 DNA</th>
<th>Detection of CyHV-3 Transcript</th>
<th>Detection of CyHV-3 Antigen after cohabitation</th>
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<tbody>
<tr>
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<td>Ide (Leuciscus idus)</td>
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<td>NT</td>
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*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 asymptotically [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-inflammatory 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
Introduction: Cyprinid herpesvirus 3

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 \textit{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 \textit{in vitro} nor for virulence \textit{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 \textit{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 \textit{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 \textit{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).
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Introduction: Cyprinid herpesvirus 3


Introduction: Cyprinid herpesvirus 3


Introduction: Cyprinid herpesvirus 3


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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

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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; Diaz-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.
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<td></td>
<td>Bonobo-HV</td>
<td>LOC100970108 (S)</td>
<td>XP_003804206.1</td>
<td>1/0</td>
<td>169 (18)</td>
<td>Bonobo</td>
<td>94.3%</td>
<td>(Arrand et al., 1981)</td>
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<tr>
<td>Rhesus lymphocryptovirus</td>
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<td></td>
<td>RhLCV</td>
<td>BCRF1 (S)</td>
<td>AAK50412</td>
<td>1/0</td>
<td>177 (29)</td>
<td>Macaque</td>
<td>97.2%</td>
<td>(Franken et al., 1996)</td>
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<td>Baboon lymphocryptovirus</td>
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<td></td>
<td>BaLCV</td>
<td>vIL-10 (S)</td>
<td>AAF23949</td>
<td>1/0</td>
<td>171 (24)</td>
<td>Baboon</td>
<td>91.6%</td>
<td>(Franken et al., 1996)</td>
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<tr>
<td><strong>Macavirus</strong></td>
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<tr>
<td>Ovine herpesvirus 2</td>
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<td></td>
<td>OvHV-2</td>
<td>Ov2.5</td>
<td>AAX5040</td>
<td>5/4</td>
<td>182 (26)</td>
<td>Sheep</td>
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<td><strong>Percavirus</strong></td>
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<td>EHV-2</td>
<td>ORF E7 (S)</td>
<td>AAC13857</td>
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<td>179 (18)</td>
<td>Horse</td>
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<tr>
<td>Cyprinid herpesvirus 3</td>
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<td></td>
<td>CyHV-3</td>
<td>ORF134</td>
<td>ABG42961</td>
<td>2/1</td>
<td>179 (17)</td>
<td>Common Carp</td>
<td>26.9%</td>
<td>(Aoki et al., 2007)</td>
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<tr>
<td>Anguillid herpesvirus 1</td>
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<td>AngHV-1</td>
<td>ORF25</td>
<td>AFK23521</td>
<td>1/0</td>
<td>165 (19)</td>
<td>European eel</td>
<td>34.3%</td>
<td>(van Beurden et al., 2010)</td>
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Table 1. Features of vIL-10s.

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

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 built using MEGA (JTT+G 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-[ILMVs]-[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 LacmIL-10 (Jenkins et al., 2008a) and CyHV-3 (Iinoue 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 LacmIL-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, LacmIL-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, LacmIL-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
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 STAT3 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 lipopolysaccharide (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., 2000).
Introduction: Viral Interleukin 10

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, LAcnvIL-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 LAcnvIL-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
kidsneys 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


Introduction: Viral Interleukin 10


Introduction: Viral Interleukin 10


Introduction: Viral Interleukin 10


Introduction: Viral Interleukin 10


inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI.

*Proc Natl Acad Sci U S A* 88, 1172-1176.


Table S1. Sequences used to build trees

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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


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](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](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

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 phylogenetically 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 \textit{in vitro} nor for virulence \textit{in vivo}

\textit{Veterinary Research} (2013), 44:53

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

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\textsuperscript{2}Proteomic and Microbiology, CISMa, University of Mons, Place du parc 20 B-7000 Mons, Belgium
\textsuperscript{3}Delphi Genetics SA, rue Antoine de Saint-Exupéry, 5, B-6041 Gosselies, Belgium
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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.
The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo

Ping Ouyang1, Krzysztof Rakus1, Maxime Boutier1, Anca Reschner1, Baptiste Leroy2, Maygane Ronsmans1, Guillaume Fournier1, Sophie Scohy3, Bérénice Costes4, Ruddy Wattiez2 and Alain Vanderplasschen1*

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 reveal 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
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 Poxxviridae 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 Poxxviridae 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. 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 minimal 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% CO2. The CyHV-3 FL strain was isolated from the kidney of a fish that died from in vitro nor for virulence in common carp.

Experimental section: Chapter 1
Table 1 Primers and probes.

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**Primers for amplification of recombination cassettes**

| H1-gaK-H2 cassette | 134 gaK F | ATGTTCCTTGCAGTCTAACCG |
| | 134 gaK R | CGACCATCTTCTGCTCAGCAGG |
| | | CACCCGACCTTTCGACGCTG |
| | | CTGTTGACAAATATGATCAGC |
| | | TCAATGTTTGCAGCTTTTTC |
| | | TCTTTTGACGTCTTTTGGAG |

| H1-ORF134-H2 cassette | H1F | GCTCATCAATGCAGCAGCA |
| | H2R | CAAGCCATATCTCCGATG |

**Primers and probes for real-time TaqMan PCR quantification of CyHV-3 genome**

| CyHV-3 ORF89 | KHV-86F | GACGCCGAGACCTTGG |
| | KHV-163R | CGGTTCTTATTTTTGTCCTGGT |
| | KHV-109P | (6FAM)CCCTCCTTGGCTCAGACCACTGBHQ1 |
| Carp glucokinase | CgGluc-162F | ACTGCGAGTCAGGACGATG |
| | CgGluc-230R | TAGGATTGGCAGGACGAC |
| | CgGluc-185P | (6FAM)AAAGCCAGTGCAAAATGCTGACCCAGAC |

**Primers for RT-qPCR analysis of carp gene expression**

| 40S | 40S-F | CGTGTTGAGCATCGTT |
| | 40S-R | TAGGACATGACCTCTG |

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

Experimental section: Chapter 1
Experimental section: Chapter 1

Table 1 Primers and probes. (Continued)

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Underlined: 50bp corresponding to CyHV-3 sequence.

in data-dependent AutoMS (2) mode with a scan range of 100–2,800 m/z, three precursors, 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 Ion searches of the NCBI nr 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 vertibrate 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 (galK) positive/negative selection in bacteria as described previously [34]. The first recombination process (galK positive selection) consisted to replace ORF134 by galK resulting in the FL galK plasmid. Recombination was achieved using the H1-galK-H2 recombination cassette (Figure 1b) which consisted of the galK gene flanked by 50-bp sequences homologous to CyHV-3 genome regions flanking ORF134 deletion (Figure 1a). H1-galK-H2 recombination cassette was produced by PCR (primers 134 galK F and 134 galK R) using the pgalK vector as template. Primer 134 galK F consisted of nucleotides 229836–229885 (50bp) of CyHV-3 genome and 1–24 (24bp) of the pgalK vector. Primer 134 galK 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-galK-H2 corresponding to CyHV-3 genome were used to target homologous recombination in bacteria. The second recombination process (galK negative selection) consisted to remove the galK gene (FL BAC ORF134 Del plasmid) or to replace the galK 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
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 co-habitation 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 101 to 1010 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 synthesized 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−ΔΔCt 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
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

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

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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

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

(Continued)

<table>
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<tr>
<th>Accession number</th>
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<td>50.8 65 2 0.07</td>
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<td>Tissue inhibitor of metalloproteinase 2b (D. rerio)</td>
<td>24.7 64 2 0.15</td>
<td>DSCVYLIGQTPOLL</td>
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**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 SacI 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.
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 SacI 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.
Effect of ORF134 deletion on viral growth in vitro

In order to investigate the putative effects of the recombinant 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 Challenge with FL strain. On day 0, common carp ($n = 30$ fish per tank), with an average weight of $3.8 \pm 1.5$ 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.

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 $3.8 \pm 1.5$ 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.

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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
observed for IL-1β and IFN-γ2. We observed a moderate and late (day 6 and day 8 post-infection) up-regulation of TNF-α1 and TNF-α2. 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

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**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 ≤ 0.05) observed between infected and mock-infected fish. Symbol (a) indicates statistical differences (p ≤ 0.05) observed for a specific time point between groups of fish infected with different CyHV-3 recombinants.
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 histiocytic 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 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.
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 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; EGF: Enhanced green fluorescent protein; galK: Galactokinase; HCMV: Human cytomegalovirus; IE: Immediate early; FN: Interferon; IL-10: Interleukin-10; KHV: Koi herpesvirus; L: Late; M: Multiplicity of infection; MS: Mass spectrometry; ORF: 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” (FNRIS) (FRFRS.2165, -2697). KR and AV are members of the BELVR consortium (AP, phase VII) granted by the Belgian Science Policy Office (BELSPO) (Belgium).
31. dx.doi.org/10.1186/1297-9716-44-44-53
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

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95
Preamble

Koi herpesvirus (KHSV), 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 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 galK 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 galK strain. The safety of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del galK 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 galK 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 galK 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 galK 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 galK 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 galK strain in small fish. The safety of the FL BAC revertant ORF56-57 Del pGEMT ORF134 Del galK 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 galK 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 trip 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 galK 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 galK gene using homologous recombination with ORF56-57 Del galK cassette in both FL BAC plasmid and FL BAC ORF134 Del plasmid. The galK 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 gaK 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 galK. 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 (Novartis 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

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Underlined: 50bp corresponding to CyHV-3 sequence  
Italic: sequence corresponding to *galK* 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% CO₂. 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 (galK) 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 galK 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 galK gene (positive selection) resulting in the FL BAC ORF56-57 Del galK and FL BAC ORF56-57 Del galK ORF134 Del plasmids. Recombination was performed using the ORF56-57 Del galK recombination cassette consisting of the galK gene flanked
by 50 base pair (bp) sequences homologous to CyHV-3 genome regions flanking ORF56-57 deletion. The ORF56-57 Del galK recombination cassette was produced by PCR (primers 56-57 galK F and 56-57 galK R) using the pgalK vector as template. Primer 56-57 galK 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 galK R consisted of nucleotides 99751-99800 (50 bp) of the CyHV-3 genome and nucleotides 1-24 (24 bp) of the pgalK vector. The 50 bp sequences of the ORF56-57Del galK corresponding to CyHV-3 genome were used to target homologous recombination in bacteria. The second recombination process (galK negative selection) consisted to remove the galK 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 for 18 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).
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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 shown 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 galK strain, CCB cells were co-transfected with the FL BAC ORF134 Del galK 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.
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