First annual meeting
Belgian Society for Virology
Friday, December 8, 2014
The Royal Academies for Science and the Arts
Hertogsstraat/Rue Ducale 1, Brussels

8h30	Welcome
8h35	Belgian Society of Virology – statues *Hans Nauwynck*
9h00-12h30	**Pathogenesis - Immune response**
9h00-9h45	keynote lecture "The long journey of hepatitis C virus entry into the hepatocyte" *Jean Dubuisson*
9h45-10h00	Selected talk 1 Viral semaphorin inhibits dendritic cell phagocytosis and migration but is not essential for gamma-herpesvirus-induced lymphoproliferation in malignant catarrhal fever *Francoise Myster, Leonor Palmeira, Oceane Sorel, Edwin De Pauw, Isabelle Schwartz-Cornil, Alain Vanderplasschen, Benjamin G. Dewals*
10h00-10h15	Selected talk 2 A phosphoproteome analysis at the host-respiratory syncytial virus interphase *Koen Sedeyn, Bert Schepens, Liesbeth Vande Ginste, Walter Fiers, Xavier Saelens*
10h15-10h30	Selected talk 3 A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus *Xiaoyun Yang, Lennert Steukers, Katrien Forier, Ranhua Xiong, Kevin Braeckmans, Kristien Van Reeth, Hans Nauwynck*
10h30-11h00	Coffee break
11h00-12h30	15 shotgun presentations (5+1 minutes each/without discussion) 1. Role of Gvin1 protein in cellular resistance to viral infection *Aurélie De Cock, Marguerite Kreit, Thomas Michiels* 2. IN variants retarget HIV-1 integration and are associated with disease progression *Jonas Demeulemeester, Sofie Vets, Rik Schrijvers, Madlala Paradise, Marc De Maeyer, Jan De Rijck, Thumbi Ndung'u, Zeger Debyser, Rik Gijsbers*

3. HCV-genotype 1b escapes antibody-mediated neutralization by viral transmission through peripheral blood B-lymphocytes
Isabelle Desombere, Ali Farhoudi-Moghadam, Freya Van Houtte, Lieven Verhoye, Caroline Buysschaert, Hans Van Vlierberghe, André Elewaut, Xavier Rogiers, Wilfried Swinnen, Philip Meuleman and Geert Leroux-Roels

4. Molecular sieve activity of the nuclear pore during HIV-1 nuclear import revealed by single virus analysis
Lieve Dirix, Doortje Borrenberghs, Stephanie De Houwer, Frauke Christ, Susana Rocha, Johan Hofkens, Zeger Debyser

5. Impact of MALT1 on rabies virus disease
Elodie Kip, Vanessa Suin, Jens Staal, Michael Kalai, Rudi Beyaert, Steven Van Gucht

6. Murid herpesvirus 4 ORF63 is involved in the translocation of incoming capsids to the nucleus
Muhammad Bilal Latif, Bénédicte Machiels, Alain Vanderplasschen, Laurent Gillet

7. Are the multiple functions of Theiler's virus Leader (L) protein coupled?
Michael Peeters, Thomas Michiels

8. Identification of cyprinid herpesvirus 3 (CyHV-3) envelope transmembrane proteins that are essential to viral growth in vitro
Michelle Penaranda, C. Vancsok, J. Jazowiecka-Rakus, V. Stalin Raj, A. Vanderplasschen

9. Pseudorabies virus gE causes ERK1/2 activation in primary porcine T lymphocytes and subsequent cell aggregation and migration
Maria Pontes, B. Devriendt, H.W. Favoreel

10. Sensitivity and permissivity of Cyprinus carpio to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier
Maygane Ronsmans, M. Boutier, K. Rakus, F. Farnir, D. Desmecht, F. Ectors, M. Vandecan, F. Lieffrig, C. Mélard, Vanderplasschen A.

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Ibrahim Sayed, Lieven Verhoye, Ali Farhoudi, Yannick Debing, Johan Neyts, Geert Leroux-Roels, Robert Purcell, Suzanne Emerson, Philip Meuleman

12. HIV triggers a cGAS-dependent type 1 IFN response in primary CD4+ T cells that is regulated by Vpu and Vpr
Jolien Vermeire, Anouk Van Nuffel, Ferdinand Roesch, Daniel Sauter, Hanne Vanderstraeten, Evelien Naessens, Veronica Iannucci, Alessia Landi, Wojciech Witkowski, Ann Baeyens, Olivier Schwartz, Frank Kirchhoff, Bruno Verhasselt

13. Age and strain dependent differences in the outcome of experimental infections of domestic pigs with Belgian wild boar pseudorabies virus isolates
Sara Verpoest, Ann Brigitte Cay, Herman Favoreel, Nick De Regge

14. HIV-1 integrase/Pol recruits LEDGF/p75 into viral particles
Caroline Weydert, B. Desimmie, R. Schrijvers, S. Vets, J. Demeulemeester, P. Proost, I. Paron, J. Mast, N. Bannert, R. Gijsbers, F. Christ, J. De Rijck, Z. Debyser

15. The envelope glycoprotein gp150 promotes sexual transmission of murid herpesvirus 4.
Caroline Zeippen, Alain Vanderplasschen, Philip Stevenson, Laurent Gillet

12h30-13h30 Sandwich lunch

13h30-17h00 More pathogenesis - Control of viral diseases

13h30-14h15 Control- keynote lecture
"IFN-lambda : the third life of IFN"
Thomas Michiels

14H15-14H30 Selected talk 1
The pseudorabies US3 protein interferes with presentation of the CD300a ligand phosphatidylserine and protects infected cells from NK mediated killing
Korneel Grauwet, C. Cantoni, T. Jacob, S. De Pelsmaeker, C. Claessen, M. Parodi, A. De Maria, L. Moretta, M. Vitale, H. Favoreel

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A stably expressed llama single-domain intrabody targeting Rev displays broad-spectrum anti-HIV activity
Eline Boons, Guangdi Li, Els Vanstreels, Thomas Vercruysse, Christophe Pannecouque, Anne-Mieke Vandamme, Dirk Daelemans

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Targeting a host-cell entry factor barricades antiviral resistant HCV variants from on-therapy breakthrough in human-liver mice
Koen Vercauteren, Richard Brown, Ahmed Atef Mesalam, Juliane Doerrbecker, Sabin Bhuju, Robert Geffers, Naomi Van Den Eede, Fulvia Troise, Lieven Verhoye, Thomas Baumert, Ali Farhoudi, Riccardo Cortese, Geert Leroux-Roels, Thomas Pietschmann, Alfredo Nicosia, Philip Meuleman

15h00-15h30 Coffee break

15h30-17h00 15 shotgun presentations (5+1 minutes each/without discussion)

1. Comparison of the pathogenesis of the highly passaged MCMV Smith strain with that of the low passaged MCMV HaNa1 strain in Balb/c mice upon oronasal inoculation
Shunchuan Zhang, Jun Xiang, Hans Nauwynck

2. Reduced susceptibility of CD4+ T-cells from elite controllers to HIV-1 infection reveals a block at a step between reverse transcription and integration
Paradise Madlala, Rik Gijsbers, Sofie Vets, Erik Van Wijngaerden, Rik Schrijvers, Zeger Debyser

3. Evasion of cytotoxic T cell response by Alcelaphine herpesvirus 1 genome maintenance protein.
Oceane Sorel, F. Myster, Vanderplasschen A., Dewals B.G.

4. Limited evolution and (almost) clonal growth of the yellow fever virus during lethal infection in mice
Dieudonné BuhKum, Niraj Mishra, Pieter Leyssen, Kai Dallmeier

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10. Comparison of tissue distribution and expression of the IBDV (Infectious Bursal Disease virus) VP2 protein by two recombinant herpesvirus vector vaccines
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11. Antiviral activity of Favipiravir (T-705) against HMPV, RSV and other paramyxoviruses
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Eva Malatinkova, Maja Kiselinova, Pawel Bonczkowski, Wim Trypsteen, Peter Messiaen, Jolien Vermeire, Bruno Verhasselt, Karen Vervisch, Linos Vandekerckhove, Ward De Spiegelaere

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17h00 **Reception with Belgian beers**

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Abstracts

1.

Resistance profile of the diaryltriazine non-nucleoside reverse transcriptase inhibitor and candidate microbicide UAMC01398

Kevin K. Ariën (1), Muthusamy Venkatraj (2), Johan Michiels (1), Katleen Vereecken (1), Jurgen Joossens (2), Pieter Van der Veken (2), Leo Heyndrickx (1), Jan Heeres (2), Koen Augustyns (2), Guido Vanham (1,3)

(1) Virology Unit, Institute of Tropical Medicine, Antwerp, Belgium
(2) Laboratory of Medicinal Chemistry, University of Antwerp, Antwerp, Belgium
(3) Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium

Background:

We previously selected UAMC01398 as a lead compound from a screen with 60 diaryltriazine analogues, in the context of a multi-partner program on microbicide development (FP7-CHAARM). This new NNRTI has a superior toxicity profile compared to Dapivirine (DPV) and it retains nM activity against DPV-resistant viruses. We now report on the resistance profile of this new candidate microbicide.
Methods:
Resistance was induced in dose-escalation studies and in single high dose experiments. Mutations were identified by sequencing and subsequently confirmed in IC50 experiments with site-directed mutagenesis in a pNL4.3 molecular clone. Cross-resistance to other clinical and experimental NNRTIs was studied. Finally, the replication capacity of the UAMC01398-resistant viruses was assessed.
Results:
Dose-escalation studies revealed the following mutations in the RT gene: V90I, V106A, E138K, V179M, H221Y, F227C and M230I. Full blown resistance was selected only after 150 days. At least 4 of these mutations are required in concert for resistance against UAMC01398. Cross-resistance was assessed against DPV, Etravirine, Rilpivirine (RPV), Lersivirine, MIV170, Efavirenz and Nevirapine. Only Etravirine and RPV retained partial activity (sub µM). Single high dose exposure to UAMC01398 did not select for resistant HIV. Finally, we clearly show that UAMC01398-resistant viruses are significantly less fit than wild type virus.
Conclusions:
UAMC01398 is a strong new candidate microbicide with superior toxicity, activity against DPV-resistant HIV, and a complex resistance profile that is not easily selected but similar to RPV.

2.

A stably expressed llama single-domain intrabody targeting Rev displays broad-spectrum anti-HIV activity.

Eline Boons [a], Guangdi Li[b], Els Vanstreels[a], Thomas Vercruysse[a], Christophe Pannecouque[a], Anne-Mieke Vandamme[b,c], Dirk Daelemans[a*]

[a] Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Department of Microbiology and Immunology, KU Leuven, Minderbroedersstraat 10, Leuven, B-3000, Belgium
[b] Rega Institute for Medical Research, Laboratory for Clinical and Epidemiological Virology, Department of Microbiology and Immunology, KU Leuven, Minderbroedersstraat 10, Leuven, B-3000, Belgium
[c] Centro de Malária e Outras Doenças Tropicais and Unidade de Microbiologia, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa 1349-008, Portugal

The HIV Rev protein mediates the transport of partially and unspliced HIV mRNA from the nucleus to the cytoplasm. Rev multimerizes on a secondary stem-loop structure present in the viral intron-containing mRNA species and recruits the cellular karyopherin CRM1 to export viral mRNAs from the nucleus to the cytoplasm. Previously we have identified a single-domain intrabody (Nb_{190}), derived from a llama heavy-chain antibody, which efficiently inhibits Rev multimerization and suppresses the production of infectious virus. We recently mapped the epitope of this nanobody and demonstrated that Rev residues K20 and Y23 are crucial for interaction while residues V16, H53 and L60 are important to a lesser extent.
We generated cell lines stably expressing Nb_{190} and assessed the capacity of these cell lines to suppress the replication of different HIV-1 subtypes. These cells stably expressing the single-domain antibody are protected from virus-induced cytopathogenic effect even in the context of high multiplicity of infection. In addition, the replication of different subtypes of group M and one strain of group O is significantly suppressed in these cell lines. One virus strain of subtype F (Bz163) was able to replicate in the presence of Nb_{190}. However, mutations in the epitope of Nb_{190} did not explain the resistance of Bz163 towards Nb_{190}.
We also analysed the natural variations of Rev amino acids in sequence samples from HIV-1 infected patients worldwide and assessed the effect of Nb_{190} on the most prevalent polymorphisms occurring at the key epitope positions (K20 and Y23) in Rev. We found that Nb_{190} was able to suppress the function of these Rev variants except for the K20N mutant, which was present in only 0.7% of HIV-1 sequence populations (n=4632).
Altogether, our results indicate that Nb_{190} may have broad potential as a gene therapeutic agent against HIV-1.

3.

Rational Design of a Safe and Efficacious Attenuated Recombinant Vaccine against Cyprinid Herpesvirus 3 using Prokaryotic Mutagenesis and In Vivo Imaging System

Boutier M.[1], Ronsmans M.[1], Ouyang P.[1], Fournier G.[1], Rakus K.[1], Reschner A.[1], Davison A.[2], Farnir F.[3], Bayrou C.[4], Lieffrig F.[5], Desmecht D.[4] and Vanderplasschen A.[1]

[1.]Immunology-Vaccinology, FARAH, ULg.
[2.]Centre for Virus Research, University of Glasgow, 8 Church Street, Glasgow, United Kingdom.
[3.]Biostatistics and Bioinformatics applied to veterinary sciences, FARAH, Ulg.
[4.]Pathology, FARAH, Ulg.
[5.]Laboratory of fish pathology, CER Groupe, 1 rue du Carmel, Marloie, Belgium.

Cyprinid herpesvirus 3 (CyHV-3) is the causative agent of a lethal disease in common and koi carp. Since its emergence, in the late 1990s, CyHV-3 has caused severe economic losses worldwide creating a need for a safe and efficacious vaccine. With this goal in mind, recombinant strains deleted for single gene were produced using prokaryotic mutagenesis. While producing such a recombinant deleted for ORF134, we unexpectedly obtained a clone additionally deleted for ORF56 and ORF57. Interestingly, this triple deleted recombinant replicated efficiently *in vitro*, exhibited an attenuated profile and induced an immune protection against a lethal challenge *in vivo*. To determine the role of each deletion in the phenotype of the triple deleted recombinant, a series of recombinant were produced and tested *in vivo*. These experiments revealed that the attenuated phenotype observed resulted mostly from the single deletion of ORF57.

Complementary experiments were performed to further investigate the potential of the ORF56-57 double deleted recombinant as a safe and efficacious attenuated vaccine. These experiments revealed that (i) *In vivo* imaging (IVIS) of vaccinated fish challenged with a wild type strain expressing luciferase suggested that vaccination induces an immune protection close to sterile immunity. (ii) Study of viral tropism by qPCR, IVIS, and histopathological analyses, demonstrated that the vaccine strain replicates at lower level, at later time post-infection and for a shorter period of time. (iii) Transmission studies demonstrated that there is no detectable spread of the vaccine from freshly vaccinated fish to naive fish located immediately downstream (water sharing).

Altogether, this study demonstrates the potential of CyHV-3 ORF56-57 double deleted recombinant for safe and efficacious mass vaccination of carp against CyHV-3.

4.

Characterisation of Transmitted and Non-Transmitted HIV in Index-Recipient Transmission Pairs

Bracke L[1], Willems E[1], Gall A[2], Kellam P[2], Coppens S[1], Meehan C[3], Pollakis G[4,5], Berkhout B[4], Vanham G[1], Cornelissen MTE[4], Heyndrickx L[1], Ariën KK[1].

[1] Virology Unit, Biomedical Sciences Department, Institute of Tropical Medicine, Antwerp, Belgium
[2] Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom
[3] Mycobacteriology Unit, Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[4] Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[5] Institute of Infection and Global Health/CIMI, University of Liverpool, United Kingdom

Many of the viral and host factors associated with HIV transmission are still poorly understood. In 60-80% of the mucosal infections, a single transmitted/founder virus is responsible for the establishment of a productive infection, indicating a strong genetic bottleneck upon transmission. We aim to better understand the viral factors involved during transmission by studying the genetic variability and replicative characteristics of viruses isolated from transmission pairs.
We had access to blood samples from 5 index-recipient transmission pairs of MSM. All samples were obtained shortly after transmission. Plasma was used for full genome sequencing and PBMC were cocultured with HIV negative donor PBMC by limiting dilution to obtain biological clones.
We isolated a total of 270 biological clones from the 5 transmission pairs. Nearly the complete gp120 from 7-18 clones was sequenced for each of the 10 individuals. As expected, these sequences group nicely with the plasma sequences. In only one transmission pair studied, an identical clone was found in both index and recipient. Phylogenetic analysis showed a low genetic diversity in the recipients, in contrast to a greater genetic diversity among the clones from the index patients. Sequences from recipients also showed shorter V1, V2 and V4 loops, indicating a more compact envelope compared to viruses from the index patients. We also found fewer potential N-linked glycosylation sites in three recipients compared to their indexes.
Our sequences and phylogenetic analysis confirm observations from others. Currently the replication capacity of the biological clones is assessed in dual infection/competition assays. This will allow us to rank the fitness of the clones obtained from all the transmission pairs. A selection of clones will also be tested against neutralizing antibodies and entry inhibitors. These experiments should give us an indication of which virus characteristics favour transmission.

5.

A NOVEL OPTIMIZED PROTOCOL TO STUDY VIROMES USING NEXT-GENERATION SEQUENCING

Nádia Conceição-Neto[1]; Mark Zeller[1]; Elisabeth Heylen[1]; Pieter de Bruyn[1]; João Rodrigo Mesquita[2]; Maria São José Nascimento[3]; Marc Van Ranst[1]; Jelle Matthijnssens[1].

[1]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, Leuven, Belgium
[2]Department of Animal Science, Rural Engineering and Veterinary, Polytechnic Institute of Viseu, Viseu, Portugal
[3]Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal

Viruses are the most ubiquitous and genetically diverse life forms on our planet and play an important role in various ecological niches, including the human gut. Next generation sequencing seems to be a promising tool to study viromes, but methods to quantify how many and which viral species are present in a biological sample are currently lacking.
In this study we show the development of sample-to-sequence pipeline, which includes the optimization of sequential purification, amplification and viral RNA and DNA sequencing steps, while reducing the introduction of biases in the relative abundances of viruses in a sample to a minimum. At first, a mock-virome was designed containing 10 highly divergent viruses, representing nearly all the known viral characteristics, including virion size range, virion architecture (spherical, rod-shaped, head-tail structure, presence/absence of an envelope), and genome types (RNA/DNA, linear/fragmented/circular). Subsequently, qPCR assays for each of the viruses present in this mock-virome and for bacteria (both genomic DNA and ribosomal RNA) were developed to evaluate the quantitative effects of several purification and amplification procedures. These include freeze-thawing, homogenization (to disrupt stool aggregates), centrifugation and filtration (to remove bacterial and eukaryotic cells), DNAse/RNAse treatment (to remove free floating DNA/RNA). As the amount of RNA and DNA recovered from a stool sample is too low to be sequenced directly by NGS technologies, random amplification procedures and library preparation procedures were also tested. These results were used to optimize a method that introduces the least amount of bias in the relative abundances of viruses in a biological sample and that could be used to deep sequence these samples using the Illumina Hiseq2500™ sequencing platform.
We then demonstrated the feasibility of the newly developed protocol by analyzing the virome in fecal samples from small Portuguese carnivores, such as a badger (*Meles meles*), a genet (*Genetta genetta*), a mongoose (*Herpestes ichneumon*) and an Eurasian otter (*Lutra lutra*). These analyses resulted in the identification of several highly divergent viruses, possibly belonging to new viral families/genera, such as five novel gemycircularviruses, a novel anellovirus, a novel nano-like virus and a novel nodavirus. Furthermore, we have successfully used this protocol to recover complete genome sequences from rotaviruses, coronaviruses, herpesviruses, astroviruses, paramyxoviruses, hepatitis B virus, among others.
This newly optimized virome method allows for thorough virome analyses of biological samples, opening the door to study the role of the virome in complex human diseases or for virus discovery.

6.

Protection from lethal yellow fever virus infection by a novel DNA-based live-attenuated virus vaccine

Kai Dallmeier[*1], Niraj Mishra[1], Dieudonné Buh Kum[1], Justin Julander[2] and Johan Neyts[1]

1) Laboratory of Virology and Experimental Chemotherapy, Rega Institute for Medical Research, KU Leuven – University of Leuven, Belgium
2) Institute for Antiviral Research, Utah State University, Logan, USA
*presenting author: kaihenrik.dallmeier@rega.kuleuven.be

The yellow fever virus (YFV), a mosquito-borne flavivirus, causes severe and life-threatening infections with jaundice, systemic bleeding, shock and multi-organ failure. An estimated 900 million people living in ~45 endemic countries in Africa and Latin America are at high risk. Although there is a safe and highly efficient live-attenuated prophylactic vaccine [YFV-17D, Stamaril®] available, an estimated 200,000 cases of yellow fever occur each year resulting into ~30,000 deaths. Vaccination with Stamaril® is recommended for everybody living in and traveling to the high risk regions. Prompt detection and rapid response through emergency vaccination campaigns are essential to control yellow fever outbreaks (often in remote and resource poor areas). However, the need for a proper cold-chain complicates the timely delivery of vaccines. Development of an easy and inexpensive to produce DNA vaccine would allow much faster and simplified deployment.
Using the YFV-17D vaccine strain as paradigm, we developed a novel plasmid based-system that allows efficient launching of recombinant flavivirus genomes *in vivo* directly from cloned cDNA. Here we assessed the efficacy and safety of our plasmid system when used as live-attenuated YFV-17D vaccine (DNA-YFVax) in Syrian golden hamsters that are susceptible to lethal yellow fever infection and that represent an established preclinical model for testing of yellow fever vaccines. We show that intraperitoneal delivery of DNA-YFVax as naked DNA is able to induce seroconversion. In fact, virus-neutralizing antibodies are generated with comparable kinetics and to equally high titers as in hamsters vaccinated with Stamaril®. Importantly, by using needle-free jet injection of plasmid DNA via the transdermal route, the efficacy of DNA-YFVax can further be enhanced, whereas the amount of plasmid needed can be reduced by at least a factor 10 to 100.
When immunized with 10µg of DNA-YFVax prior to challenge, hamsters with high neutralizing antibody titers had (i) undetectable viremia, (ii) normal ALT-levels and (iii) were protected from yellow fever induced disease and mortality. This resembles the protection elicited by Stamaril® which was used as a comparator.
In summary we show proof of concept that our convenient, robust and reproducible plasmid system may allow to develop a DNA vaccine for YFV at low costs without the need for eukaryotic cell cultures or embryonated chicken eggs. It will no longer require a cold-chain and might be needle-free administered. Moreover, our technology may represent a convenient platform for future engineering and manufacturing of other recombinant live-attenuated and chimeric flavivirus vaccines.

7.

A Mutation in the Hepatitis E Virus RNA Polymerase Promotes its Replication and Associates With Ribavirin Treatment Failure in Organ Transplant Recipients

Yannick Debing[1], Anett Gisa[2], Kai Dallmeier[1], Sven Pischke[3], Birgit Bremer[2], Michael Manns[2], Heiner Wedemeyer[2], Pothakamuri Venkata Suneetha[2], Johan Neyts[1]

[1] Rega Institute for Medical Research, Department of Microbiology and Immunology, University of Leuven, Belgium
[2] Department of Gastroenterology, Hepatology and Endocrinology, Center of Internal Medicine, Hannover Medical School, Germany
[3] Universitätsklinikum Hamburg-Eppendorf Medizinische Klinik I, Germany

Chronic hepatitis E is often treated with extended courses of ribavirin and treatment failure has been occasionally observed. Here we report on two transplant patients chronically infected with the hepatitis E virus (HEV) who underwent extensive ribavirin therapy, but failed to clear the virus. In one case, the viral load decreased initially but viral rebound was noted 4 months after start of treatment and consequently the patient developed fatal decompensated liver disease. The second patient experienced twice a relapse following ribavirin treatment (which was for 5 and 8 months respectively), despite undetectable virus at the end of each treatment period. The viral genome was sequenced at various time points. For both patients, a single G-to-A nucleotide substitution resulting in a G1634R mutation in the viral polymerase was associated with ribavirin failure. This mutation was engineered into a genotype 3 (gt3) subgenomic HEV replicon. The mutant replicon proved equally sensitive to the antiviral activity of ribavirin as the wild-type construct. However, the 1634R replicon (and also a 1634K mutant) replicated markedly more efficiently than the wild-type, suggesting an increased replication fitness. This was confirmed by analysis of the growth kinetics of full-length (gt3) virus. Similar results were obtained with a gt1 replicon. In direct competition assay, the full length gt3 1634R mutant was able to out-compete the wild-type virus. In conclusion, we report on a HEV polymerase mutation that is associated with ribavirin treatment failure and that results in an increased *in vitro* replication fitness.

8.

Murid herpesvirus 4 infection protects mice from the development of an anti-pneumovirus vaccine-induced TH2 immunopathology

Mickael Dourcy[1], Bénédicte Machiels[1], Caroline Zeippen[1], Jérémy Dumoulin[1], Justine Javaux[1], Daniel Desmecht[2], Alain Vanderplasschen[1], Benjamin Dewals[1], Laurent Gillet[1]

[1]Laboratory of Immunology-Vaccinology, FARAH, University of Liège, Belgium.
[2]Laboratory of Pathology, FARAH, University of Liège, Belgium.

Gammaherpesviruses are highly prevalent pathogens that establish lifelong latency. However, little is known about how these viruses imprint the immune system of their host. Here we used Murid herpesvirus 4 (MuHV-4) to investigate the impact of gammaherpesvirus infections on the development of an anti-pneumovirus vaccine-induced Th2-skewed immunopathology. Briefly, this respiratory hypersensitivity was induced in mice by a subcutaneous vaccination with formalin-inactivated antigens of pneumonia virus of mice (FI PVM) followed by an intranasal infection with wild-type PVM. We have observed that MuHV-4 infection, either before or after the FI PVM vaccination, prevented the development of the PVM-induced immunopathology while the protection against PVM infection was unaffected. This protective impact against the immunopathology was maintained over time and required pulmonary MuHV-4 replication. Altogether, these results open perspectives for vaccination against pneumoviruses and highlight that some so-called pathogens could be revealed in the end as beneficial for their host.

9.

Vaccine effectiveness estimates in preventing laboratory-confirmed mild and moderate-to-severe influenza in the Belgian population during the 2012-2013 season

Anneleen hombrouck[1], Nathalie Bossuyt[2], Viviane Van Casteren[2], Steven Van Gucht[1,3], Sophie Quoilin[2], the Belgian Network of sentinel general practitioners, the Belgian Network of sentinel hospitals, Françoise Wuillaume [2], and Isabelle Thomas [1]

[1]Scientific Institute of Public Health, Operational Directorate of Communicable and Infectious Diseases, Viral Diseases, Brussels, Belgium
[2]Scientific Institute of Public Health, Operational Directorate of Public Health and Surveillance, Brussels, Belgium
[3]Ghent University, Laboratory of Virology, Faculty of Veterinary Medicine, Ghent, Belgium

Background
The influenza season 2012-2013 in Belgium was characterized by a very high intensity and duration as compared to previous seasons. Three viruses co-circulated: A(H1N1), A(H3N2) and B with a predominance of A(H1N1) and B of Yamagata lineage.

Methods
Mild cases of influenza were monitored through a sentinel network of 112 general practitioners (GPs), which reported and sampled patients of any age who met the influenza-like-illness (ILI) case definition: sudden onset of symptoms, fever (≥38°C), respiratory and systemic symptoms.
Moderate-to-severe influenza cases were monitored through a sentinel network of 6 hospitals, which reported and sampled patients with severe acute respiratory infections (SARI), which were defined as patients of any age with an acute respiratory illness with onset within the last seven days and fever of ≥ 38°C (or history of fever) and cough or dyspnoea, and requiring hospitalisation (24h or more).
Patients with swabs taken > 7 days of symptoms onset and patients with no record of vaccination (date) or vaccination < 14 days before symptoms onset were excluded.
We used a test-negative design and compared lab-confirmed influenza-positive to influenza-negative patients with mild or moderate-to severe illness. Using logistic regression, we calculated adjusted vaccine effectiveness (AVE) by type/subtype and results were adjusted for surveillance scheme (if applicable), age group, sex and month of sample collection.

Results
During the 2012-2013 season, 1425 samples from ILI and 1001 samples from SARI patients were collected. After applying exclusion criteria, we included 1261 ILI patients and 665 SARI patients in the vaccine effectiveness (VE) analysis.
Overall trivalent influenza vaccine (TIV) adjusted VE against laboratory-confirmed influenza (mild and moderate-to-severe cases) was estimated 39% (95%CI 17%-54%). TIV adjusted VE against influenza A(H1N1) was 48% (95%CI 20%-67%) and influenza B was 44% (95%CI 19%-61%). Data power was not sufficient to calculate TIV adjusted VE for influenza A(H3N2).
When comparing laboratory-confirmed mild influenza to moderate-to-severe influenza, overall TIV adjusted VE against mild influenza was estimated 42% (95%CI 12%-63%) and against moderate-to-severe influenza was 35% (95%CI 0.7%-57%). TIV adjusted VE against mild influenza A(H1N1) alone was 51% (95%CI 0.6%-65%) and against moderate-to-severe influenza A(H1N1) was 47% (95%CI 0.3%-71%). TIV adjusted VE against mild influenza B alone was 56% (95%CI 27%-75%). The adjusted VE estimate against moderate-to-severe influenza B was lower but the effect was not significant.

Conclusions

Estimation of VE was feasible in Belgium for the 2012-2013 season, because the number of ILI and SARI samples collected in our routine surveillance systems were high due to the intensity of the epidemic. The data suggest a moderate protection by TIV against mild and moderate-to-severe influenza during the 2012-2013 season.

10.

Comparison of tissue distribution and expression of the IBDV (Infectious Bursal Disease virus) VP2 protein by two recombinant Herpesvirus vector vaccines

INGRAO Fiona*, RAUW Fabienne*, LAMBRECHT Bénédicte*, VAN DEN BERG Thierry*

*Department of Avian Virology and Immunology, CODA-CERVA, rue Groeselenberg 99, 1180 Bruxelles

Infectious bursal disease (IBD) is an avian viral disease leading to important economic losses in the poultry industry worldwide. This very contagious disease is characterized by lymphoid organs destruction, especially the bursa of Fabricius, inducing an immunosuppression. Affected chickens showed increased susceptibility to infection with opportunistic pathogens and a suboptimal vaccines response. In order to control the disease, several vaccinations methods have been developed. Because attenuated IBD virus (IBDV) vaccines may lead to partial lymphoid organs destruction, the use of safer vectors was required. One of them is the recombinant Herpesvirus of turkey (HVT) in which a foreign gene was introduced. The HVT is widely used as a vaccine against the Marek Disease virus because it is safe and poorly sensitive to the interference with maternal derived antibodies. Moreover, the HVT is secure to be injected *in ovo* or in one day-old chicks. Because of its cell-associated replication, it induces the development of a persistent viremia and a long-term protection. Regarding IBDV, the gene of concern is encoding for the immunogenic structural protein VP2. The efficacy of the bivalent recombinant HVT-IBD to immunize chickens against the IBDV is well known. However, the immune mechanisms involved in the protection conferred by this type of vaccines against IBD and the tropism for the host tissues are still poorly documented. With the aim of studying the vaccine take and the replication kinetics, we first intended to investigate the early distribution of two HVT-IBD vectors in several organs (bursa, feathers, lungs, spleen and peripheral blood lymphocytes) after day-old vaccination by developing two real-time PCR: one specific for the VP2 and the other one for the glycoprotein B of the HVT. The results of this experiment showed that the targeted organs, ie bursa, feathers, lungs, spleen and peripheral blood lymphocytes, are colonized by the HVT-IBD 5 days post vaccination. An interesting observation was the accumulation of the HVT-IBD vector in the feather pulp until at least 12 days after vaccination. In the other organs, the viral DNA load tends to fluctuate during the two first weeks post-inoculation before decrease. These findings were similar for both vaccines studied. To complete these results, we managed to compare the production of the VP2 by the HVT-IBD by labeling this protein for detection by immunofluorescence on an infected cells culture.

11.

Antiviral activity of Favipiravir (T-705) against HMPV, RSV and other Paramyxoviruses

D. Jochmans[1], S. van Nieuwkoop[2], B. Martina[2], P. Lexmond[2], S. Herfst[2], J. Neyts[1], and B. van den Hoogen[2]

[1] Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[2] Erasmus MC, Rotterdam, The Netherlands

Background
The total clinical impact of Paramyxoviruses such as Parainfluenza virus (PIV), RSV and HMPV infections together argues for the development of antiviral therapies with broad-spectrum activity against paramyxoviruses. Favipiravir (T-705) is a broadly active small molecule active against several RNA viruses and is presently in clinical evaluation for the treatment of Influenza viruses. In the current study we evaluated the *in vitro* activity of favipiravir against a number of Paramyxoviruses and the *in vivo* activity against HMPV infection in a hamster model.

Methods
The 50% effective concentrations (EC_{50}) for favipiravir against Measles virus, Newcastle Disease Virus, Avian Pneumovirus, PIV, RSV and HMPV *in vitro* was determined by dose-response studies in Vero cells. To evaluate the efficacy of favipiravir against HMPV *in vivo*, Syrian golden hamsters were orally treated twice a day with 0-50-100-200-300-400 mg/kg/day, starting one day prior to challenge with HMPV until day 4, when animals were euthanized. Infectious viral titers in lungs and nasal turbinate's were determined with titrations in Vero cells and the presence of viral RNA was determined with qRT-PCR.

Results
Favipiravir treatment inhibited replication of all paramyxoviruses tested *in vitro*, with EC50 values ranging between 2.5 and 12.5 ug/ml. Treatment with 400 mg/kg/day resulted in 100% protection of the lungs in HMPV challenged hamsters and in significant decreased viral titers in the upper and lower respiratory tract in animals treated with 200-300 mg/kg/day, compared to mock treated animals. Viral RNA remained detectable in the upper and lower respiratory tract of all treated hamsters. Only in hamsters treated with 400 mg/kg/day a decrease in viral genome titers was observed.

Conclusions
We demonstrate antiviral activity of favipiravir against a broad range of paramyxoviruses *in vitro* and against HMPV *in vivo*. The observation that favipiravir treatment had a significant effect on infectious viral titers, with limited effect on viral RNA titers, is in agreement with its proposed viral mutagenesis mode of action. With the reported antiviral activity of T-705 against Influenza- and Picorna- viruses, this small molecule would be a promising broad range antiviral drug candidate to limit the viral burden of all these respiratory viruses.

12.

R030987 is a broad-spectrum, early-stage inhibitor of rhinovirus replication with a mode of action that targets the inner capsid protein VP4.

Céline Lacroix[a], Alain Cobut[c], David Franco[a], Kim Donckers[a], Antonia Volny-Luraghi[c], Emilie Blanche[b], Arnaud Marchand[c], Patrick Chaltin[b,c], Johan Neyts[a] and Pieter Leyssen[a]

[a]Laboratory of Virology and Experimental Chemotherapy, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[b]Centre for Drug Design and Discovery, KU Leuven, Leuven, Belgium
[c]Centre for Innovation and Stimulation of Drug Discovery, CISTIM Leuven vzw, Leuven, Belgium

Rhinoviruses (genus enterovirus, family *Picornaviridae*) infections not only cause common colds in otherwise healthy people, but also lead to asthma- and COPD exacerbations. No antiviral drugs for human use are currently available.
In a large scale CPE-based antiviral screening campaign, R030987 was identified as a potent and selective inhibitor of rhinovirus 14 (HRV14) replication with an 50% effective concentration (EC_{50}) of 5±2 µM. The compound proved also active (EC_{50} between 3 and 29 µM) against a panel of 15 different rhinoviruses representative for species HRV-A and HRV-B. Time-of-drug addition studies revealed that R030987, akin to the capsid binder pleconaril, inhibits virus replication at an early stage of the viral replication cycle. However, R030987 retained wild-type activity against pleconaril-resistant HRV14 isolates and thermostability assays revealed that the compound does, unlike pleconaril, not protect against heat inactivation of the virus. Three drug-resistant virus variants were obtained independently by clonal selection. In a CPE-based assay they proved three- to seven-fold less sensitive to the compound than the parent viruses. All three of these virus variants were shown to have acquired a mutation at position 35 of the internal capsid protein VP4: A35S (2/3) and A35V (1/3). Residue VP4 A35 was found to be highly conserved in the VP4 protein of all 15 of the different rhinovirus serotypes that were included.
Boththe A35S and A35V mutation were reverse-engineering into an infectious clone of HRV14 ($HRV14_{IC}$). In a CPE reduction assay, the mutants proved respectively 3- and 4-fold less susceptible to the antiviral effect of R030987. This confirms that the mutated amino acid position, which in the crystal structure of the virion appears to be located in a highly unstructured stretch of amino acids, is indeed involved in the mechanism of action of this inhibitor. Further elucidation of the precise molecular mechanism by which this class of compounds acts and exploration of the structure-activity relationship will provide a deeper insight into the (different) role(s) of VP4 at the early stages of viral replication. In conclusion, we demonstrate, to the best of our knowledge, for the first time that VP4 may offer a novel target for the development of broad-spectrum inhibitors of rhinovirus replication.
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13.

Accurate Quantification of Episomal HIV-1 2-LTR Circles Using Optimized DNA Isolation and Droplet Digital PCR

Malatinkova Eva[a], Kiselinova Maja[a], Bonczkowski Pawel[a], Wim Trypsteen[a], Messiaen Peter[a], Vermeire Jolien[b], Verhasselt Bruno[b], Vervisch Karen[a], Vandekerckhove Linos[a], De Spiegelaere Ward[a]

a) HIV Translational Research Unit, Department of Internal Medicine, Faculty of Medicine and Health Sciences, Ghent University and University Hospital Ghent, Ghent, Belgium
b) Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Medicine and Health Sciences, Ghent University and University Hospital Ghent, Ghent, Belgium

ABSTRACT
In HIV-1 infected patients on combination antiretroviral therapy, the detection of episomal HIV-1 2-LTR circles is considered as a marker for ongoing viral replication. PCR based quantification of 2-LTR circles is hampered due to its low abundance and a critical step of sample pre-PCR processing has not yet been sufficiently evaluated in patient derived samples. We compared two sample processing procedures to more accurately quantify 2-LTR circles using droplet digital PCR. Episomal HIV-1 2-LTR circles were either isolated by genomic DNA isolation or by a modified plasmid DNA isolation, allowing separation of the small episomal DNA from chromosomal DNA and normalization to a spiked internal control plasmid. A linear correlation of both methods was observed in the dilution series (R^2=0.974) and in the patient derived samples with 2-LTR numbers above 10 copies per million peripheral blood mononuclear cells (n=17), (R^2=0.671). Furthermore, Bland-Altman analysis revealed an average agreement between the methods within the 27 samples with detectable 2-LTR circles by both methods (bias: 0.39 ± 1.27 $\log_{10}$). The plasmid DNA isolation resulted in more accurate quantification in the low ranges and a higher recovery of 2-LTR templates per isolate compared to the total genomic DNA isolation. PCR inhibition caused by high genomic DNA load limits the amount of sample input after genomic DNA isolation, which impacts sensitivity and accuracy. Therefore, 2-LTR circles quantification in HIV-1 infected patients is more accurate by using a modified plasmid DNA isolation method, enabling to process more blood cells, thus enhancing quantification accuracy and sensitivity. An improved quantification of 2-LTR circles will contribute to the better understanding of ongoing replication in the HIV reservoir of patients on cART.

14.

The A239G mutation in motif A compensates for the lethal K159R mutation in the active site of the Coxsackievirus B3 RNA-dependent RNA polymerase and increases the susceptibility of this virus to the antiviral effect of T-705

Rana Abdelnabi, Leen Delang, Pieter Leyssen, Johan Neyts

Rega Institute for Medical Research, University of Leuven, Belgium
Minderbroedersstraat 10, 3000 Leuven

Favipiravir (T-705), is a broad-spectrum antiviral agent that was originally discovered as an inhibitor of influenza A virus replication. In the cell, T-705 is metabolized to its ribofuranosyl 5'-triphosphate form, which was shown to be a competitive inhibitor for the incorporation of ATP and GTP by the RNA-dependent RNA polymerase (RdRp). We recently demonstrated that T-705 also inhibits the *in vitro* replication of the chikungunya virus (CHIKV) and protects mice against CHIKV-induced mortality (Delang *et al*., J Antimicrob Chemother 2014). Low-level T-705-resistant CHIKV variants were selected. A K291R mutation in the F1 motif of the RdRp was shown to be responsible for the observed resistance to T-705. Interestingly, K291 is highly conserved in positive single-stranded RNA viruses.

The Coxsackie B3 virus (CVB3) is 4 fold less sensitive to T-705 than CHIKV. Introduction of a K-to-R mutation at the corresponding position (K159R) in the F1 motif of the CVB3 RdRp resulted in a lethal phenotype. Thus the CVB3 RdRp has a different tolerance for mutations at this position than CHIKV. However, when the R159 CVB3 variant was transfected in Vero cells without antiviral pressure, a second mutation in the RdRp, i.e. A239G, emerged that was able to rescue the replication fitness of the R159 mutant. This amino acid position is located in motif A and is strictly conserved in the RdRp of all enteroviruses. In addition, both the reverse engineered CVB3 R159-G239 and G239 variant demonstrated to be more susceptible to the antiviral effect of T-705 than the wild-type virus. Interestingly, the CVB3 G239 mutant has been reported as a low RdRp fidelity variant (Gnädig NF *et al*., Proc Natl Acad Sci USA.2012). Therefore, the increased susceptibility of the double mutant and G239 variants to T-705 may be related to the increased rate of nucleotides misincorporation caused by A239G mutation.

15.

Mouse-to-mouse transmission of norovirus is efficiently blocked by prophylaxis with the viral polymerase inhibitor 2'-C-methylcytidine

J. Rocha-Pereira, D. Jochmans, J. Neyts

KU Leuven - University of Leuven, Rega Institute for Medical Research, Leuven, Belgium

Human noroviruses are a major cause of foodborne illness, accountable for 50% of all-etiologies outbreaks of acute gastroenteritis. These outbreaks are quite extensive and can disturb the functioning of health institutions, leading to closure of hospital wards and causing life-threatening infections in long-term care facilities. To add to this large burden of disease, noroviruses are responsible for significant mortality among children in developing countries. It is therefore a pressing matter to develop strategies for the treatment and/ or prophylaxis of norovirus infections.

We previously reported that the viral polymerase inhibitor 2'-C-methylcytidine (2CMC) efficiently protects against murine norovirus (MNV)-induced diarrhea and mortality in AG129 (deficient in alpha/beta and gamma interferon receptors) mice (Rocha-Pereira et al., 2013, J Virol 87:11798-805). We here established a MNV-transmission model using genogroup V (murine) norovirus in AG129 mice in order to assess the ability of an antiviral molecule to prevent or reduce transmission of (murine) norovirus either when given to the infected (seeder) mice or to the uninfected (sentinel) mice. In this model, MNV was efficiently transmitted from infected mice to (100% of) sentinels with which they had been in direct contact. To estimate the viral inoculum which would need to be transmitted from mouse to mouse to cause MNV-induced disease, we determined the 50% infectious dose to be ~270 $CCID_{50}$ (via oral gavage). When the sentinel mice were treated with 2CMC, 100 mg/kg/day for 5 days, these sentinels developed no symptoms during or after treatment with 2CMC, remaining healthy for the entire duration of the experiment. Thus, prophylaxis with 2CMC was able to completely prevent the sentinels from developing MNV-induced disease. Treatment of infected mice with 2CMC also reduced viral shedding and markedly reduced transmission to uninfected sentinels.

In conclusion, we demonstrated that a small molecule inhibitor of norovirus replication is able to efficiently prevent the transmission of MNV from infected to uninfected mice. Such inhibition may be an important tool to control norovirus outbreaks, in particular, by protecting yet uninfected individuals against the infection (for example, in hospital wards and long-term care facilities) and for treating chronically infected immunocompromised patients. Although it should be emphasised that a murine model as the one presented here does not recapitulate the pathology/ pathogenesis and epidemiology of human norovirus infections, our findings provide an important first piece of evidence that antivirals may have a role in the control of norovirus outbreaks. The frequency and high burden of norovirus hospital outbreaks highlight the need for additional measures (other than sanitary). The use of antivirals should hence be considered for the prevention of norovirus infections in high-risk populations.

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16.

PHYLOGENETIC ANALYSIS OF FIV STRAINS FROM NATURALLY INFECTED CATS IN BELGIUM AND THE NETHERLANDS

Inge DM Roukaerts[1], Sebastiaan Theuns[1] , Elien RL Taffin[2], Sylvie Daminet[2], and Hans J Nauwynck[1]

Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium[1]
Department of Small Animal Medicine and Clinical Biology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium[2]

Feline immunodeficiency virus (FIV) is a major pathogen in feline populations worldwide. Virus strains circulating in domestic cats are subdivided into different phylogenetic clades (A to E), based on the genetic diversity of the V3-V4 region of the *env* gene. For 36 FIV strains isolated in Belgium and The Netherlands, a phylogenetic analysis of the V3-V4 *env* region, and a variable region in the *gag* gene was made. Genomic DNA was isolated from freshly drawn blood of naturally infected cats. Fragments of the *gag* and *env* gene were amplified by PCR and sequenced. All new *gag* sequences clustered together with previously known clade A FIV viruses, confirming the dominance of clade A viruses in Northern Europe. The same was true for the new *env* sequences, with only one sample of an unknown *env* subtype. In conclusion, a low genetic variability was seen for *gag* fragments and V3-V4 *env* sequences of FIV strains found in Belgium and The Netherlands. This is in accordance with the more uniform variability of FIV already reported in Northern Europe. However, the unknown *env* fragment indicates that new introductions of FIV strains from unknown subtypes occur which may increase genetic variability in time. Because a correlation between subtypes and pathogenicity could not be made so far, it might be interesting to add other gene fragments to the classification system. This may lead to a better understanding of determinants of virulence.

17.

Clinical sentinel surveillance of equine West Nile fever, Spain

Claude Saegerman[1], Ana Alba-Casals[2], Ignacio García-Bocanegra[3], Fabiana Dal Pozzo[1], Gaby van Galen[4]

[1] Research Unit of Epidemiology and Risk Analysis applied to veterinary science (UREAR-ULg), Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liege, Liege, Belgium; E-Mail: claude.saegerman@ulg.ac.be; fdalpozzo@ulg.ac.be

[2] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, Barcelona, Spain; E-Mail:ana.alba@cresa.uab.cat

[3] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Córdoba-Agrifood Excellence International Campus (ceiA3), Córdoba, Spain; E-Mail: v62garbo@uco.es

[4] Large Animal Clinic, Internal Medicine and Surgery, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; E-Mail: gaby@equinespecialists.eu

Running title: Clinical surveillance of equine West Nile fever

Corresponding author:
Claude Saegerman, Research Unit in Epidemiology and Risk Analysis Applied to Veterinary Sciences (UREAR-ULg), Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liege, Boulevard de Colonster 20, bât. B42, B-4000, Liege, Belgium. Phone: +3243664579, fax : +3243664261, e-mail : Claude.Saegerman@ulg.ac.be

Summary
West Nile fever (WNF) is a viral zoonotic infection caused by a mosquito-borne flavivirus of the *Flaviviridae* family. According to a comparative study, the passive surveillance of horses by equine veterinarians appeared to be the most cost-effective system in the European context of WNF. Clinical data issued from a passive epidemiosurveillance network from September 2010 to December 2011 on horses in Spain were statistically compared and used to develop a predictive diagnostic decision tree, both with the aim to improve the early clinical detection of WNF in horses. Although clinical signs were variable in horses affected by WNF, four clinical signs and the month of occurrence were identified as useful indicators to distinguish between WNF related and unrelated cases. The signs that pointed out a presumptive diagnosis of WNF in horses were cranial nerves deficits, limb paralysis, photophobia and nasal discharge. Clinical examination of horses with neurological signs that are not vaccinated against WNV could provide important clues for the early clinical detection of WNF, and therefore, serve as an alert for possible human viral infections. The study of the clinical pattern of WNF in horses is of importance to enhance awareness and better understanding, and to optimize surveillance designs for clinical detection of WNF in horses in advance of epidemic activity affecting humans.
Keywords: West Nile fever, equine, virus, vector-borne disease, clinical epidemiology, data mining.

18.

Protection and mechanism of action of a novel human Respiratory Syncytial Virus vaccine candidate based on the extracellular domain of Small Hydrophobic protein

Bert Schepens[1,2], Koen Sedeyn[1,2], Iebe Rossey[1,2], Marianne Liesbeth VanDen Ginste[1,2] Sarah De Baets[1,2],, Michael Schotsaert[1,2], Kenny Roose[1,2], Lieselot Houspie[3], Marc Van Ranst[3] ,Brian Gilbert[4], Pedro Piedra[4,6], Xavier Saelens[1,2]

[1]Department for Molecular Biomedical Research, VIB, 9052 Ghent, Belgium
[2]Department of Biomedical Molecular Biology, Ghent University, 9052 Ghent, Belgium
[3]Laboratory of Clinical Virology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[4]Molecular Virology and Microbiology and [6]Pediatrics, Baylor College of Medicine, Houston TX 77030, USA
[5]Department of Molecular Cell Biology, Vrije Universiteit, 1081 BT Amsterdam, The Netherlands

Infections with human respiratory syncytial virus (HRSV) occur globally in all age groups. In young infants HRSV is the leading cause of severe lower respiratory tract disease, hospitalization and an important cause of death. Therefore, a prophylactic vaccination strategy that can prevent disease caused by HRSV is high on the priority list of health officials. Despite decades of research vaccination strategies focusing on the induction of neutralizing antibodies have not being successful. This might in part be attributed to the remarkable capacity of HRSV to escape from neutralizing antibodies and hence re-infect throughout life, despite the absence of significant antigenic drift. We have developed a novel vaccination strategy that is based on antibodies that do not neutralize but engage alveolar macrophages to target and remove HRSV infected cells. We demonstrate that a vaccine based on the extracellular domain (SHe) of the HRSV small hydrophobic protein (SH), reduces viral replication in HRSV challenged laboratory mice and in cotton rats. Remarkably, even though immune serum from SHe-immunized animals lacks virus neutralizing activity, protection can be transferred into naïve recipient animals by such serum. We also show that protection by SHe-specific antibodies depends on a functional IgG receptor compartment with a major contribution of FcγRI and/or FcγRIII. In contrast, preliminary results suggest that these Fcγ receptors do not play a major role in the protective activity of palivizumab. Using a conditional cell depletion method we provide evidence that alveolar macrophages but not NK cells are required for protection by SHe-specific antibodies. We show that in contrast to HRSV virions infected cells can readily be recognized by SHe-specific antibodies. These antibodies can engage macrophages to phagocyte RSV infected cells in vitro. Hence we propose a mechanism by which the humoral immune response elicited by SHe-based vaccination controls HRSV replication by promoting the clearance of HRSV infected cells. Because newborns are the major target group for RSV, we are currently investigating whether maternal SHe-vaccination of mice can protect offspring against RSV infections via antibody transfer. Preliminary results indicate that SHe-specific antibodies are readily transferred from the vaccinated mother to the offspring and can significantly hamper RSV replication in these pups. Thus, by targeting SHe as a vaccine antigen, it is possible to induce immune protection against HRSV by a mechanism that differs from the natural immune response.

19.

Tick-borne encephalitis virus (TBEV) – Seroprevalence study for TBEV antibodies in bovine sera in Belgium: a risk-based screening

Suin V.[1*], Roelandt S.[2*], Lamoral S.[1], Riocreux F.[3], Van der Heyden S. [4], Van der Stede Y.[2,5], Lambrecht B.[6], Caij B.[7], Brochier B.[1], Roels S.[4], Van Gucht S.[1,8]

[1]National Reference Centre of TBEV and Rabies, Viral diseases, Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium.
[2]Unit for Coordination of Veterinary Diagnosis, Epidemiology and Risk Assessment (CVD-ERA), Operational Directorate of Interactions and Surveillance, Veterinary and Agrochemical Research Centre (VAR-CODA-CERVA), Brussels, Belgium.
[3]Unit for Data Management and Analysis (DMA), Operational Directorate of Interactions and Surveillance, Veterinary and Agrochemical Research Centre (VAR-CODA-CERVA), Brussels, Belgium.
[4]Unit of Pathology and Prionology, Operational Directorate of Interactions and surveillance, Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium.
[5]Department of Immunology, Faculty of Veterinary Medicine, University of Ghent, Merelbeke, Belgium.
[6]Unit of Avian Virology and Immunology, Operational Directorate of Virology, Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium.
[7]Unit of Enzootic and (Re-)emerging Diseases, Operational Directorate of Virology, Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium.
[8]Ghent University, Laboratory of Virology, Faculty of Veterinary Medicine, Ghent, Belgium
* both authors contributed equally

Tick-borne encephalitis virus (TBEV) is the most important arthropod-borne virus in Europe. The Western subtype of this pathogenic neurotropic flavivirus is vectored by *Ixodes ricinus*. Tick-borne encephalitis has become a considerable public health risk in several European countries, with currently 3000 hospitalized cases per year. The risk of TBEV-introduction into Belgium remains high and the presence of infected wildlife in Belgium is suspected. Domestic animals such as cattle can serve as excellent sentinels for TBEV-surveillance in order to install an early warning surveillance component for this emerging zoonotic disease of public health importance.
A serological screening was performed on Belgian cattle (n=650), selected from the 2010 Belgian national cattle surveillance serum bank. The three most Eastern provinces of Belgium, which are geographically situated closest to known and/or recently emerging TBEV-endemic, were targeted.
Bovine sera were tested by gold standard TBEV seroneutralisation test (SNT), based on the rapid fluorescent focus inhibition test (RFFIT) protocol. Using a conservative >1/15 cut-off titer for SN test, 17 bovines were seropositive and six had borderline results (1/10 < titer < 1/15). The accuracy of the SNT was confirmed in a mouse inoculation test and by West Nile virus and Rabies virus serology. There was a positive correlation between the neutralizing antibody titer, determined by SN, and the median survival time in mice inoculated intranasally with a mix of virus and serum.
The overall bovine TBEV-seroprevalence in the targeted area was estimated between 2.6 and 4.3% and freedom could no longer be substantiated. Bovines with borderline results were often located close to confirmed seropositive animals. The geographical locations roughly coincided with the known Belgian hot spots for Lyme disease.
This risk-based serological survey in cattle confirms the presence of infected foci in Belgium for the first time. Given the relevance of TBEV for the food chain through consumption of unpasteurized milk and cheese and through its considerable public health burden in other European countries, further surveillance in cattle, other sentinels, ticks and humans at risk is recommended to further determine the location and size of endemic foci and the risk for public health.

20.

Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses

Sebastiaan Theuns[1], Elisabeth Heylen[2], Mark Zeller[2], Inge D.M. Roukaerts[1], Lowiese M.B. Desmarets[1], Marc Van Ranst[2], Hans J. Nauwynck[1] and Jelle Matthijnssens[2]

[1]University of Ghent, Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Laboratory of Virology, B-9820 Merelbeke, Belgium
[2]KU Leuven - University of Leuven, Department of Microbiology and Immunology, Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, B-3000 Leuven, Belgium

Group A rotaviruses (RVAs) are an important cause of diarrhea in young pigs and children. An evolutionary relationship has been suggested to exist between pig and human RVAs. This hypothesis was further investigated by phylogenetic analysis of the complete genomes of six recent (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], and G9P[23]) and one historic (G1P[7]) Belgian pig RVA strains, and of all completely characterized pig RVAs around the globe.
In contrast to the large diversity of genotypes found for the outer capsid proteins VP4 and VP7, a relatively conserved genotype constellation (I5-R1-C1-M1-A8-N1-T7-E1-H1) was found for the other 9 genes in most pig RVA strains. VP1, VP2, VP3, NSP2, NSP4 and NSP5 genes of porcine RVAs belonged to genotype 1, which is shared with human Wa-like RVAs. However, for most of these gene segments, pig strains clustered distantly from human Wa-like RVAs, indicating that viruses from both species have entered different evolutionary paths. However, VP1, VP2 and NSP3 genes of some archival human strains were moderately related to pig strains. Phylogenetic analysis of the VP6, NSP1 and NSP3 genes, as well as amino acid analysis of the antigenic regions of VP7 further confirm this evolutionary segregation. The present results also indicate that the species barrier is less strict for pig P[6]-strains, but that chances for successful spread of these strains in the human population are hampered by the better adaptation of pig RVAs to pig enterocytes. However, future surveillance of pig and human RVA strains is warranted, as reassortment of pig gene segments in human RVA strains may lead to the spread of reassortant virus strains in the human population.

21.

The immunity raised by recent European subtype 1 porcine reproductive and respiratory syndrome virus (PRRSV) strains allows spatial expansion of highly virulent East European subtype 3 PRRSV strains

Ivan Trus[1], Ilias S. Frydas[1], Caroline Bonckaert[1], Vishwanatha R.A.P. Reddy[1], Yewei Li[1], Lise K. Kvisgaard[2], Lars E. Larsen[2], Hans J. Nauwynck[1]

[1]Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.
[2]National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark.

Three subtypes of porcine reproductive and respiratory syndrome virus type 1 (PRRSV-1) are present in Europe. Subtype 1 strains are mainly present in the European Union. They cause severe reproductive problems (late abortion/early farrowing, stillbirth, weakborn piglets) but no or little general and respiratory problems. Subtype 2 and 3 strains are circulating in former member states of the Sovjet Union (e.g. Belarus). They are causing severe general and respiratory problems (high fever during 2-3 weeks, dyspnea and hyperpnea) and reproductive problems (similar to those described for subtype 1 strains). Despite the fear for the introduction of subtype 2 and 3 strains in the European Union, the spatial distribution of these PRRSV-1 subtypes in Europe has been surprisingly stable in the past. In the present study, we evaluated the potential of three West European subtype 1 PRRSV strains (07V063, isolated in 2007; 13V091, isolated in 2013 and 13V117, also isolated in 2013) to provide protection against the East European subtype 3 PRRSV strain Lena. Three groups of 11-week-old pigs were inoculated with either PRRSV 07V063 or PRRSV 13V091 or PRRSV 13V117. A fourth group was not inoculated (control group). Seven weeks later, the pigs of the four groups were challenged with the East European subtype 3 PRRSV strain Lena. Clinical, virological and serological parameters were monitored upon challenge with PRRSV Lena. The number of fever days was higher ($P < 0.05$) in the control group (7.6 ± 1.7 days) compared to animals from the three other groups (07V063-immunized: 4.0 ± 1.2 days, 13V091-immunized: 4.6 ± 1.1 days, 13V117-immunized: 4.0 ± 2.9 days). Reduction of respiratory disease and nasal shedding (mean AUC and mean peak values) was more pronounced in animals immunized with the 07V063 strain ($P < 0.05$) than in the two other groups. In all immunized groups, protection was characterized by a shorter ($P < 0.05$) viremia period (control group: 13.0 ± 4.8 days, 07V063-immunized: 5.4 ± 0.9 days, 13V091-immunized: 5.0 ± 0.0 days, 13V117-immunized: 5.8 ± 1.1 days). Our results indicate that immunity elicited by inoculation with subtype 1 PRRSV strains can partially protect against antigenically divergent subtype 3 strains. The lower protection level at the respiratory tract (portal of entry) elicited by recently isolated subtype 1 PRRSV strains may form a danger for subtype 3 strains to spread in the European Union.

22.

Comparison of Illumina MiSeq and Ion Torrent PGM next generation sequencing for influenza A virus quasispecies analysis

Silvie Van den Hoecke[1,2], Judith Verhelst[1,2], Marnik Vuylsteke[3] and Xavier Saelens[1,2]

[1] Inflammation Research Center, VIB, Zwijnaarde (Ghent), 9052, Belgium
[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, 9000, Belgium
[3] Gnomixx, Onafhankelijkheidslaan 38, Ghent, 9000, Belgium.

Determining the composition of the influenza viral quasispecies requires an accurate and sensitive sequencing technique and analysis pipeline. We compared the suitability of two benchtop next-generation sequencers: the Illumina MiSeq sequencing-by-synthesis and the Ion Torrent PGM semiconductor sequencing technique. We first validated the accuracy and sensitivity of both sequencers using plasmid DNA and spiking-in experiments of mutant plasmid. Illumina MiSeq sequencing reads were one and a half times more accurate than those of the Ion Torrent PGM. To evaluate the suitability of the two techniques for determining the quasispecies composition of influenza A virus, we generated plasmid-derived PR8 virus. We optimized an RT-PCR protocol to obtain uniform coverage of all eight genomic RNA segments. Sequence analysis of these amplicons revealed that more sequencing errors (mostly indels) were generated by the Ion Torrent PGM. Based on RT-PCR and intrinsic sequencing errors, we found that the detection limit for reliable recognition of variants in the viral genome required a frequency of 0.5% or higher. Most of the variants in the PR8 genome were present in hemagglutinin, and these mutations were detected by both sequencers. Our approach underlines the power and limitations of two commonly used next-generation sequencers for the analysis of influenza gene diversity.
Contact: silvie.vandenhoecke@irc.vib-ugent.be

23.

Characterization by immunogold electron microscopy of a recombinant Newcastle disease virus vaccine expressing the avian influenza H5 protein at its surface

Olivier Vandersleyen[A], Mieke Steensels[A], Eveline Verleysen[B], Jan Mast[B] and Bénédicte Lambrecht[A]

[A] Avian Virology and Immunology Unit, Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Ukkel, Brussels, Belgium
[B] Electron Microscopy Unit, Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Ukkel, Brussels, Belgium

Newcastle diseases virus (NDV) has been widely used as a vector for expression of heterologous proteins in the field of vaccination. Previously, a live recombinant LaSota NDV vaccine expressing a chimeric H5 subtype hemagglutinin of an avian influenza virus (rNDV-H5) has been tested for its vaccinal potency in our lab. A protective effect of the vaccine against a highly pathogenic avian influenza HPH5N1 could be demonstrated and has been studied in the context of interaction with maternal derived antibodies (MDA). It was observed that the NDV and H5 MDA have a different impact on the rNDV-H5 vaccine efficacy. To get a better understanding of the obtained protection and the induced immune response by the rNDV-H5 vaccine, the determination of the entry mode of the rNDV-H5 vaccine is required. To achieve this goal, the characterization of the glycoprotein presentation at the surface of the vaccine has been done by immunogold electron microscopy. First, the expression of the H5 protein at the surface of the rNDV-H5 vaccine has been checked. Subsequently, the surface expression of the different NDV glycoproteins has been compared between the parental LaSota strain and the recombinant virus to determine if the presence of the H5 protein at the surface of the rNDV-H5 vaccine has an impact on the surface expression of the fusion (F) and hemagglutinin-neuraminidase (HN) NDV glycoproteins.
The immunogold experiment confirmed H5 expression at the surface of the rNDV-H5 vaccine as gold particles were observed at its surface. However, the quantity of H5 proteins at the surface was variable from one virion to another and approximately one third of the recombinant virions did not present any H5 at their surface, which could have an important impact on immune induction and therefore on the vaccine efficiency. Moreover, it was also shown that the presence of the H5 protein at the surface of the recombinant virus influences the surface expression of both the NDV F and HN proteins. Both F and HN were present in reduced amount at the surface of the recombinant virus compared to the parental strain. This last finding could also have an impact on the entry of the vaccine and therefore on its efficiency. However, further investigation remains necessary to fully understand the entry mechanism of the rNDV-H5 vaccine.

24.

Quantifying the replication-competent fraction of the proviral load in HIV$^+$ patients.

W. van Dyck, E. Willems, L. Heyndrickx, D. Berkvens, E. Florence, M. van Frankenhuijsen, K. Fransen, M. Mangelschots, M. Claus, P. Nys, K. Ariën, G. Vanham

Institute of Tropical Medicine, Nationalestraat 155, B - 2000 Antwerpen

Abstract

BACKGROUND

Presently used treatment against Human Immunodeficiency Virus (HIV), referred to as combination antiretroviral therapy (cART), can suppress the viral life cycle and allow recovery from disease progression. Treatment success is evaluated by measuring the plasma viral load and the CD4 count. However, HIV is a lentivirus that integrates its genome into CD4$^+$ cells. The integrated DNA, the provirus, is not influenced by current cART and therefore this proviral reservoir is not routinely monitored during treatment. The replication competent fraction (RCF) of the proviral reservoir is a potential source of new virion production. This fraction, expressed as Infectious Units per Million CD4$^+$ T cells (IUPM) is on average smaller than 1% and can be measured by a viral outgrowth assay (VOA). Since RCF is responsible for viral rebound after treatment interruption and therefore the primary hurdle for a functional cure, we believe that measuring the RCF in treated patients is a valuable tool for the evaluation of treatment and disease progression.

GOAL

We aim to develop an optimized VOA for the measurement of RCF in treated patients starting from the VOA protocol described previously by Siliciano et al. (PLoS Pathogen, 2013). We will use the data on the RCF of 6 treated patients to look for correlations between clinical data and IUPM.

METHODS

We evaluated; our protocols for viral outgrowth, CD4 isolation methods and several indicator cells in 10 untreated HIV$^+$ patients and established a new VOA protocol. In the end, we measured RCF in 6 treated patients and compared the resulting IUPM to clinical data in order to search for correlations.

RESULTS

As expected, untreated patients had a higher RCF (mean IUPM: 95 +/- 117) than treated patients (mean IUPM: 0.32 +/- 0.56). In addition, half of the treated patients (3/6) showed RCF on the limit of detection (0.3 IUPM). Subsequently, we showed (Spearman's correlation coefficient of 0.713; p 0.002) that the RCF determined by VOA correlates with the total proviral load determined by a commercieally available qPCR (BioCentric, Bandol, France). We did not see a correlation of RCF with plasma viral load, CD4 count and time on treatment.

DISCUSSION

The correlation between RCF and total proviral load was only significant using both treated and untreated patients combined while there was no correlation at all using treated or untreated patients separately. Therefore we believe that a PCR-based measurement of total cellular DNA only cannot replace the functional VOA to quantify the RCF. Sensitivity of VOA is still suboptimal and reproducibility has not been tested yet. Therefore we feel that more work is needed to increase sensitivity and reproducibility of this VOA protocol before it can be used in other studies.

25.

Targeting a host-cell entry factor barricades antiviral resistant HCV variants from on-therapy breakthrough in human-liver mice

Koen Vercauteren[1], Richard J.P. Brown[2], Ahmed Atef Mesalam[1], Juliane Doerrbecker[2], Sabin Bhuju[4], Robert Geffers[4], Naomi Van Den Eede[1], Fulvia Troise[6], Lieven Verhoye[1], Thomas Baumert[7,8], Ali Farhoudi[1], Riccardo Cortese[5], Geert Leroux-Roels[1], Thomas Pietschmann[2,3], Alfredo Nicosia[6,9], Philip Meuleman[1]

[1]Center for Vaccinology, Ghent University, Gent, Belgium.
[2]Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany
[3]German Centre for Infection Research (DZIF), partner site Hannover-Braunschweig
[4]Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[5]Keires AG, Basel, Switzerland
[6]Department of Molecular Medicine and Medical Biotechnology, University of Naples Frederico II, Naples, Italy
[7]Institut National de la Santé et de la Recherche Médicale, U1110, Strasbourg, France
[8]Université de Strasbourg, Strasbourg et Pole Hépato-digestif, Hopitaux Universitaires de Strasbourg, Strasbourg, France
[9]CEINGE, Naples, Italy

After host cell entry of the hepatitis C virus (HCV), viral replication takes place involving both viral and cellular proteins. Direct-acting Antivirals Agents (DAAs) inhibit HCV infection by targeting viral proteins that play an essential role in the replication process. This type of compounds is successfully being used to treat chronic HCV infections. However, therapy failure caused by the emergence of DAA-resistance associated variants (RAVs), resulting from the high level of HCV replication and the low fidelity of the viral RNA polymerase, remains a reasonable concern. Therefore, the conserved host factors used by the virus for its propagation seem interesting targets for antiviral intervention, complimentary to the action of DAAs. Presumably, future anti-HCV therapies will be a combination of drugs targeting distinct steps of the HCV life cycle.

In this study we addressed whether an entry inhibitor could prevent on-therapy breakthrough of DAA-RAVs by impeding their spread from the hepatocytes in which they are selected, thereby improving virologic response. We observed that HCV infected mice with a humanized liver receiving monotherapy of the protease inhibitor (PI) Ciluprevir (BILN-2061) rapidly experienced viral breakthrough. Using Illumina deep sequencing technology we confirmed the manifestation of drug resistant mutations in the HCV NS3/4A protease domain upon viral rebound. In contrast, mice that were treated with a combination of Ciluprevir with the entry inhibitor mAb1671, an SR-BI-specific human monoclonal antibody, did not experience on-treatment viral breakthrough. These data indicate that by preventing intrahepatic viral spread, mAb1671 precludes the dissemination of PI-resistant mutants during combination therapy thereby markedly improving virologic response.
In this study we provide preclinical *in vivo* evidence that addition of an entry inhibitor to an anti-HCV DAA regimen is an excellent strategy to prevent spread of DAA-resistant viruses and therapeutic failure due to the on-therapy rebound of DAA-RAVs.

26.

Development of assays to assess potential inhibitory activity on *in vitro* and *in vivo* Zika virus replication.

Joanna Żmurko[a], Suzanne Kaptein[a], Rafael Elias[a,b], Johan Neyts[a]

[a] Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[b] Immunopharmacology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais , Belo Horizonte, Brazil .

The Zika virus (ZIKV) is a member of genus flavivirus within the family of Flaviviridae. The virus was first isolated in 1947 from a rhesus monkey in the Zika Forest of Uganda and is now widely prevalent in Africa and South East Asia. It is transmitted by mosquitoes and has been isolated from a number of *Aedes* species. ZIKV causes a disease known as Zika fever characterized by headaches, maculopapular rash, fever, malaise, conjunctivitis, and arthralgia. A ZIKV outbreak in 2007 in Micronesia resulted in about ~5.000 infected individuals and during explosive outbreak in French Polynesia in 2013 an estimated 28.000 cases sought medical care (approximately 11% of the population). Currently there is neither a vaccine, nor a specific therapy for the treatment of infections with the ZIKV.

We here report the establishment of assays for the screening of inhibitors against the ZIKV and for the further confirmation of hits. These include a cytopathic effect reduction (CPE) assay, a viral RNA yield reduction assay (based on quantification of viral RNA) and an assay for the quantification of viral antigens (to which end a pan-flavivirus antibody was used). *In vitro* ZIKV replication is inhibited by interferon-alpha, ribavirin and nucleoside analogues such as 2'-*C*-methylcytidine, 7-deaza-2'-*C*-methyladenosine, favipiravir [T-705, a drug developed for the treatment of infections with the influenza virus] and its analogue T-1105. Next, we established an infection model in mice. To this end we employed 129/Sv mice deficient in both alpha/beta (IFN-α/β) and gamma (IFN-γ) interferon receptors. Intraperitoneal inoculation of these mice with 10^{2}-10^{5} PFU of ZIKV resulted, within 13 days, in the development of virus-induced disease (ruffled fur, lower limbs paralysis, body weight loss, reduced activity); mice that lost >20% of body weight were euthanized. The viral load in blood and organs was quantified. To validate the animal model for antiviral studies, the effect of the nucleoside analogue 2'-*C*-methylcytidine on viremia development and virus-induced disease and mortality was monitored. Initial experiments indicate that 2'-*C*-methylcytidine reduces the viremia, but is not able to significantly delay the virus-induced morbidity. Detailed analysis will be presented.

27.

Identification of host factors involved in HIV-1 Vpr mediated cell death

Baeyens A, Van Nuffel A, Landi A, Iannucci V[*], Vermeire J, Naessens E, Vanderstraeten H, Verhasselt B.

Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University, Ghent, Belgium
[*]Present address: Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York,
NY, USA

HIV infection is characterized by a gradual CD4+ T cell number decline. TheVpr protein is thought to be involved in this, due to its in vitro toxic potential and possible role in the viral life cycle. This is further supported by in vivo observations that Vpr deleted viruses are less pathogenic to the infected host.
So far, the contribution of Vpr to HIV-associated cell death and the mechanisms of Vprinduced cell death have not been clearly elucidated.

In this study, we are evaluating a list of known and putative interaction partners of Vpr, of which some were already evaluated in cell lines.
These proteins are active at different steps of cell death and survival and could provide insights on the mechanisms of Vpr induced cell death in T cells.
Among these, members of the mitochondrial pore complex ANT, BAX and VDAC1 were selected, together with other mitochondrial proteins. In addition, some genes functionally established to be important for infectivity of macrophages and dendritic cells and to induce G2 arrest, were included (e.g. CUL4A and VPRBP).

Primary $CD4^+$ T cells and different T cell lines were either infected with HIV-1 or transduced with a vector expressing Vpr protein. The roleof the selected host factors on HIV replication and survival was assessed by means of shRNA-mediated knock-down.
From these, hits were selected based on novelty, reproducibility and absence of toxic side-effects. These proteins will further be evaluated in functional assays, looking into specific Vpr functions, e.g. apoptotic markers, G2 cell cycle arrest or mitochondrial depolarization. Preliminary results will be shown, including some unexpected consequences of knock-down.

28.

Development of an *in vitro* HIV latency model utilising primary central memory T cells and a replication-competent virus

Bonczkowski Pawel[1], De Spiegelaere Ward[1], Martins Laura[2], Bosque Alberto[2], Malatinkova Eva[1], Kiselinova Maja[1], Trypsteen Wim[1], Vermeire Jolien[3], Witkowski Wojciech[3], Van Nuffel Anouk[3], Verhasselt Bruno[3], Planelles Vicente[2], Vandekerckhove Linos[1]

1 Ghent University and University Hospital Ghent, Department of Internal Medicine, Ghent, Belgium
2 Division of Microbiology and Immunology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84112 USA
3 Ghent University and University Hospital Ghent, Department of Clinical Chemistry and Microbiology, Ghent, Belgium

Background
HIV latency research will benefit from *in vitro* latency models that closely mimic *in vivo* latency. The T_{CM} model described by Bosque and Planelles generates high numbers of resting latently infected cells. The initially described model is based on a wild type env deficient virus co-transfected with an HIV env to make a single round virus. Many experiments in our laboratories led to high levels of background infection and massive cell death. These were reminiscent of a spreading infection.
Methods
To assess the replication potential of the virus used in the T_{CM} model, the original complemented virus as well as the culture supernatants of the T_{CM} model were used to transduce Jurkat T-cells and fresh T_{CM}. Subsequently, PCR and deep sequencing were performed to identify recombination as a likely source of replication competent virus. Finally, an adapted model using replication-competent HIV strains carrying EGFP/HSA reporter genes and including cell density manipulations in the presence of ART were assessed to provide a more standardized T_{CM} model.
Results
A spreading infection in the Jurkat and primary cells suggested the presence of a replication competent virus as a cause of background infection and cell death. Experiments with VSV-G and backbone only constructs excluded contamination as the source of this virus. PCR and deep sequencing analysis confirmed the presence of an intact env in the infected cells, indicating that the env deleted constructs recombine with the env sequence used for co-transfection.
An alternative approach using replication competent NL4.3 virus with an IRES-EGFP construct in combination with antiretroviral drugs to block spreading infection proved useful for medium throughput screening of anti-latency compounds. This approach leads to up to 35% of latent infection in the presence of 15% of productive infection. The wild type NL4.3 combined with CD4 expression based cell sorting and cell crowding after infection to promote cell-to-cell HIV transmission can be used for high-throughput assays. Depletion of CD4(-) cells leaves a population of up to 20% latently infected cells with little to no active infection.
Conclusions
Recombination between sequences used to generate single-round vectors in the original model introduces bias to the data and impacts data interpretation. Our study shows that the issue of recombination can be omitted. As an alternative strategy, we propose to use replication competent virus in combination with ARV to generate latently infected T_{CM}.

29.

Limited evolution and (almost) clonal growth of the yellow fever virus during lethal infection in mice

Dieudonné Buh Kum[#], Niraj Mishra[#], Pieter Leyssen, Johan Neyts and Kai Dallmeier[*]

Laboratory of Virology and Experimental Chemotherapy, Rega Institute for Medical Research, KU Leuven – University of Leuven, Belgium
[#]equal contribution
*presenting author: kaihenrik.dallmeier@rega.kuleuven.be

The yellow fever virus (YFV) is prototype member of the genus *Flavivirus*, a group of small enveloped (+)-sense RNA viruses. Other important flaviviral pathogens causing severe and life-threatening infections are the dengue viruses, the Japanese encephalitis virus and the West Nile virus. Due to their huge global disease impact, there is active surveillance and monitoring of their evolutionary history. By contrast, very little is known about the genetic diversity and intrahost evolution of flaviviruses. In analogy to other RNA viruses that behave as viral quasispecies (such as the closely related hepatitis C virus) diversification may occur during flaviviral replication in the vertebrate host. This is especially important given the fact that flaviviruses, that have an error-prone replication strategy, are transmitted by arthropod vectors that inoculate minute amounts of viruses in the vertebrate.
We assessed the effect of yellow fever virus infection (derived from the commercially available vaccine Stamaril®) in AG129 mice (interferon-receptor deficient). Mice were infected intraperitoneally with different viral inocula. A very uniform morbidity was observed that was markedly independent of the viral dose inoculated requiring euthanasia at about 12 days after infection. Clinical signs were a ruffled fur, flaccid hind limb paralysis, a hunched posture and rapid weight loss requiring euthanasia. The virus was isolated from the brain, which is consistent with viral invasion of the CNS and virus-induced encephalitis. Only when a viral inoculum as low as 0.1 pfu was used, animals developed (a similar) disease at a later time (>17 days p.i.). When mice were inoculated with <0.01 pfu, no obvious disease was observed; yet YFV-17D specific antibodies developed in these animals.
Viruses were isolated from the brains of mice succumbing to YFV-17D induced encephalitis and were plaque purified. Intriguingly, no changes were observed in plaque morphologies. About ten plaque-purified virus clones were sequenced per mouse brain and these sequences were compared to the Stamaril® derived inoculum. Overall, the brain derived YFV-17D RNA populations were quite homogenous, with error-rates ranging from 1 − 8 changes per clone and Shannon entropies of 0.002 − 0.01. We next used a novel and proprietary plasmid-based system (that we recently developed) to launch clonal YFV-17D (DNA-YFVax) following simple injection of naked DNA in the animals. YFV-17D derived from the brain of at least some of the mice that had been infected with the DNA-YFVax was calculated to have error rates and Shannon entropies of zero. Thus the heterogeneity detected in Stamaril® infected mice is the likely consequence of pre-existing heterogeneity in the life-attenuated vaccine. In summary we show that, in contrast to many other RNA viruses, YFV-17D (and likely other flaviviruses as well) do not necessarily grow to highly diverse viral quasispecies during amplification in their vertebrate hosts. This may possibly reduce antigenic drift and likewise lower the risk of resistance against antiviral drugs.

30.

Risk of emergence of a hyperpathogenic bovine leukemia virus by mutation of a single envelope N-linked glycosylation site

Alix de Brogniez[1], Amel-Baya Bouzar[1], Jean-rock Jacques[1], Nicolas Gillet[1], Isabelle Callebaut[2], Michal Reichert[3] and Luc Willems[1]

[1] GxABT and GIGA, University of Liege, Gembloux and Liege, Belgium
[2] MPMC, UMR 7590, CNRS, Université Pierre & Marie Curie, Paris, France
[3] Department of Pathology, National Veterinary Research Institute, Pulawy, Poland

Corresponding author e-mail: Alix.deBrogniez@doct.ulg.ac.be

Pathogens have co-evolved with their host to allow efficient replication and transmission without inducing excessive pathogenicity that would indirectly impair their persistence. This is exemplified by the bovine leukemia virus (BLV) model that induces lymphoproliferative disorders in ruminants only after extended latency periods of several years. In principle, the equilibrium reached between the virus and its host could be disrupted by emergence of more pathogenic strains. Intriguingly, this type of hyperpathogenic BLV strain could never been isolated in vivo nor designed in vitro. Using reverse genetics of an infectious molecular provirus, we have now identified a N-linked envelope glycosylation site that limits viral replication and pathogenicity. Onset of this particular mutation may thus represent a potential threat associated with emergence of hyperpathogenic BLV strains and possibly of new variants of the related primate T-lymphotropic viruses.

31.

Role of Gvin1 protein in cellular resistance to viral infection

Aurélie De Cock – Marguerite Kreit - Thomas Michiels

VIRO Unit, De Duve Institute, U.C.L., Belgium

Primary mouse neurons readily responded to type I IFN treatment by upregulating the expression of interferon stimulated genes (ISGs) such as Mx protein or Oasl2. However, in contrast to other cells, IFN-treated neurons failed to acquire a strong resistance to subsequent infection by Theiler's virus (TMEV).
Microarray studies identified a set of 15 genes that were upregulated by IFN treatment in mouse embryonic fibroblasts (MEFs) and in L929 cells but that were not upregulated or much less expressed in IFN-treated primary neurons.
One of these genes that is not expressed in primary mouse neurons is Gvin1. This gene encodes a very large GTPase (280 kDa), inducible by both type I and type II IFNs. In the mouse, Gvin1 is a member of a family of at least six similar genes and is located on chromosome 7. Surprisingly, the homologue of Gvin1 in human is a pseudogene.
We observed, by qRT-PCR, that Gvin1 is constitutively expressed in many organs of the mouse such as lungs, heart, spleen or liver, and that expression of this gene can be increased by IFN treatment *in vivo*. Yet, expression of Gvin1 was low in the central nervous system.
Knock-down of Gvin1 in IFN-treated and in untreated fibroblast slightly increased TMEV and VSV infection but strongly increased MuHV4 infection. Preliminary data showed that the reexpression of Gvin1 in those cells decreased by 2 fold TMEV infection and that the GTP binding domain of Gvin1 was required for its antiviral activity.
Surprisingly, Gvin1 had a strong impact on transfection kinetics of a reporter vector expressing luciferase. Knock-down of Gvin1 in fibroblast triggered a regular increase of luciferase activity up to 48h post transfection, whereas the signal dropped at this time in control fibroblast.
In line with the influence of Gvin1 on transfection, we observed that Gvin1 knock-down had a strong influence on the organization of actin filaments.
In conclusion, Gvin1 plays a role in cellular resistance to viral infection. Our hypothesis is that the protein affects the entry of virus into the cell by modifying the actin cytoskeleton. The lack of Gvin1 expression in primary neurons after IFN treatment can contribute to the high sensitivity of these cells to viral infection.

32.

IN Variants Retarget HIV-1 Integration and are Associated with Disease Progression

Jonas Demeulemeester[1,2], Sofie Vets[1], Rik Schrijvers[1], Paradise Madlala[1,3], Marc De Maeyer[2], Jan De Rijck[1], Thumbi Ndung'u[3], Zeger Debyser[1,*], Rik Gijsbers[1,*]

[1] KU Leuven, Laboratory for Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, Leuven, Belgium
[2] KU Leuven, Laboratory for Biomolecular Modeling, Department of Chemistry, University of Leuven, Leuven, Belgium
[3] HIV Pathogenesis Program, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

Background: Distinct integration patterns of different retroviruses, including HIV-1, have puzzled virologists for over 20 years. A tetramer of the viral integrase (IN) assembles on the two viral cDNA ends, docks onto the target DNA (tDNA) to form the target capture complex (TCC) and catalyzes viral genome insertion into the host chromatin.
Methods: We combined structural information on the Prototype Foamy Virus TCC with conservation in retroviral IN protein alignments to determine aa-tDNA base contacts. We generated HIV-1 variants based on the observed variability at these positions, assessed replication capacities and performed integration site sequencing to reveal their integration preferences. Finally, we examined their effect on disease progression in a chronic HIV-1 subtype C infection cohort.
Results: We identified retroviral IN amino acids affecting molecular recognition in the TCC and resulting in distinct local tDNA nucleotide biases. These residues also determine the propensity of the virus to integrate into flexible tDNA sequences. Remarkably, natural polymorphisms IN_{S119G} and IN_{R231G} retarget viral integration away from gene dense regions. Precisely these variants were associated with rapid disease progression in a chronic HIV-1 subtype C infection cohort.
Conclusions: Our findings reveal how polymorphisms at positions corresponding to HIV IN_{119} and IN_{231} affect local as well as global integration site targeting. Intriguingly, these findings link integration site selection to virulence and viral evolution but also to the host immune response and antiretroviral therapy, since HIV-1 IN_{119} is under selection by HLA alleles and integrase inhibitors.

33.

HCV-genotype 1b escapes Antibody-Mediated Neutralization by Viral Transmission through Peripheral Blood B-lymphocytes

Isabelle Desombere[1], Ali Farhoudi-Moghadam[1], Freya Van Houtte[1], Lieven Verhoye[1], Caroline Buysschaert[1], Hans Van Vlierberghe[2], André Elewaut[2], Xavier Rogiers[3], Wilfried Swinnen[4], Philip Meuleman[1,§] and Geert Leroux-Roels[1,§]

[1]Center for Vaccinology, Ghent University, 9000 Ghent, Belgium. Departments of [2]Gastroenterology and Hepatology, and [3]General and Hepato-Biliary Surgery - Liver Transplantation Service, Ghent University Hospital, Ghent, Belgium. [4]Medisch-Sociaal Opvangcentrum voor druggebruikers (MSOC), Ghent City, Ghent, Belgium. [§]equal contribution

Although infections with the hepatitis C virus (HCV) mainly cause liver diseases, extra-hepatic manifestations of the disease – especially B-cell dysfunctions - were reported. In recent years evidence has accumulated that HCV infects not only hepatocytes but also peripheral B-lymphocytes, generating an extra-hepatic HCV-reservoir. Nevertheless, the *in vivo* infectious potential of patient-derived B-cells has not been demonstrated yet. To address this question, sera and bead-sorted B-lymphocytes from 6 gt1a- and 6 gt1b-infected HCV-patients were injected separately in human liver uPA-SCID mice. Results show that purified B-lymphocytes from 6 gt1b-infected chronic patients are able to transmit HCV infection to chimeric uPA-SCID mice and install a robust *de novo* infection. B-cells from gt1a-infected patients lack this capacity. This genotype-specific difference in viral B-cell transmission most likely resides in the capacity of HCV gt1b - and not HCV gt1a - to replicate in B-lymphocytes. Since plasma from 5 out of 6 chronically gt1b-infected patients is non-infectious, we demonstrate *in vivo* that gt1b-infected B-cells are a source of infectious virions. To elucidate the origin of the B-cell passaged virions in gt1b-patients, the viral quasispecies distributions (based on complete E1E2 sequences) in human plasma, B-cells and uPA-SCID plasma were compared. Unlike HCV in human plasma, viruses found in mouse plasma following B-cell mediated infection have a very restricted diversity (mostly 1 variant) and most closely resemble replication-competent virions residing inside B-cells. Using *in vitro* (HCVpp) and *in vivo* (viral challenge of chimeric mice pretreated with patient-derived IgG) model systems, we further demonstrate that passaged HCV-virions are recognized and neutralized by autologous IgG.
B-lymphocytes are able to transmit HCV to hepatocytes *in vivo* and provide a vehicle for HCV gt1b to persist in the presence of neutralizing antibodies.

34.

Molecular sieve activity of the nuclear pore during HIV-1 nuclear import revealed by single virus analysis

Lieve Dirix§‡, Doortje Borrenberghs§‡, Stéphanie De Houwer‡, Frauke Christ‡, Susana Rocha§, Johan Hofkens§ and Zeger Debyser‡

‡ Laboratory for Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Kapucijnenvoer 33, 3000 Leuven, Belgium
§ Laboratory for Photochemistry and Spectroscopy, Department of Chemistry, KU Leuven, Celestijnenlaan 200F, 3001 Heverlee, Belgium

The human immunodeficiency virus type 1 (HIV-1) has the ability to infect non-dividing cells. Hence, the viral particle is challenged to cross the nuclear membrane by means of active transport through the nuclear pore. Studies of the nuclear import mechanism of the HIV-1 preintegration complex (PIC) are typically based on the biochemical analysis of lysates of infected cells. Such studies are limited because of the difficulty to discriminate between authentic PIC proteins and non-PIC proteins. Furthermore, only averaged information can be deduced from a pool of virions, which is problematic knowing that only a small subset of HIV virions are functional and reach the nucleus. Therefore studies on the single virus level are required. Here we introduce an optimized version of an established fluorescence microscopy-based method (Albanese et al., 2008) that fulfils this requirement. Visualisation of cells infected with engineered HIV-1 particles containing the fusion protein integrase-eGFP (IN-eGFP) allows an accurate estimation of the fluorescence intensity of each single IN-eGFP complex. Therefore, the dynamics of IN composition per complex can be studied during the journey of the PIC from the cell membrane until arrival in the nucleus. We show that nuclear complexes contain less IN in comparison with PICs in the cytoplasm, and that this is a LEDGF/p75 independent process. Next, LEDGINs, small molecule inhibitors of the interaction between integrase and LEDGF/p75 (Christ et al., 2010), prevent the reduction in the number of IN molecules at the nuclear membrane. This observation can explain why LEDGIN-induced multimerization prevents nuclear import (Desimmie et al., 2013). Together with results from time-course experiments, our data indicate that the nuclear pore is acting as a molecular filter, only tolerating PICs with a specific composition and/or size to pass through. The ability to study PIC composition at the single virus level provides the long-sought approach to unravel nuclear import of HIV-1.

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35.

A murine model for co-infection using the human blood fluke *Schistosoma mansoni* and γ-herpesvirus

Dougall A. M.[1], Rolot, M.[1], Vanderplasschen, A.[1], Dewals, B.[1]

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

Schistosomiasis caused by a numerous species of parasitic helminths (flukes) is a severe disease affecting some of the world's poorest populations. More than 200 million people are infected worldwide, most of which occur in sub-Saharan Africa. Geographically, *S. mansoni* overlaps with the human γ-herpesviruses such as the Kaposi's sarcoma-associated herpesvirus, also responsible for severe malignancies. The strongly regulated Th2-type immune response generated during infection by *S. mansoni* may jeopardize or improve the host's ability to generate effective immunity against co-infecting pathogens, such as viruses.
Traditionally host-pathogen interactions are studied in isolation of other infections but this is rarely the case in the natural environment. The study of helminth-viral co-infection is an emerging area of research. In recent months studies have demonstrated the helminth Th2 dominant environment can be beneficial to viruses. As was the case for murine γ-herpesvirus (MuHV-4) latency where infection with either *Heligmosomoides polygyrus* or *S. mansoni* eggs administration was able to reactivate murine γ-herpesvirus (MuHV-4) *in vivo*. Interleukin-4 was shown to promote viral replication and block of the antiviral effects of interferon- γ.
We have trialled two approaches to monitor helminth-virus co-infection using the MuHV-4 model. The first a *S. mansoni* egg model, whereby eggs are injected intravenously circulating to the lungs to induce granulomas and typical Th2-type responses. This is followed by intranasal infection of a MuHV-4-Luc (luciferase reporter). Alternatively, we naturally infected mice with *S. mansoni* cercariae until migration and pairing of adults in the mesentery venules (6-7 weeks post-infection). At this stage when eggs have induced granulomas and strong the Th2-type responses MuHV-4 luc is administered. In stark contrast to above, our studies show a significant reduction of MuHV-4 replication within the lungs at day 5 and 7 post-infection. This is confirmed by *in vivo* imaging and virus titration. Additionally, weight loss caused by MuHV-4 infection was significantly reduced at day 8 post-infection for the co-infected group in the model of natural infection. We present an examination of viral latency, antibody isotypes, cytokine responses and cell populations within the lung during MuHV-4 infection. This project provides valuable insights into the complexity of viral co-infection with helminths and may uncover undescribed immunological pathways that could inhibit or protect against viral infection.

36.

Novel Sp1 transcription factor binding sites in the Human T-cell leukemia/lymphoma virus type 1 Long Terminal Repeat negatively regulate its sense promoter activity.

Sylvain FAUQUENOY[1], Gwenaëlle ROBETTE[1], Anna KULA[1], Nadège DELACOURT[1], Caroline VANHULLE[1], Christian SCHWARTZ[2], Arsène BURNY[1], Olivier ROHR[2], Benoit VAN DRIESSCHE[1] and Carine VAN LINT[1].
Presenting author: Sylvain.Fauquenoy@ulb.ac.be
Corresponding author: cvlint@ulb.ac.be

[1]Service of Molecular Virology, Institut de Biologie et de Médecine Moléculaires (IBMM), Université Libre de Bruxelles (ULB), Rue des Professeurs Jeener et Brachet 12, 6041 Gosselies, Belgium.
[2]Institut Universitaire de Technologie Louis Pasteur de Schiltigheim, University of Strasbourg, Allée d'Athènes 1, 67300 Schiltigheim, France.

Background. Human T-lymphotropic Virus 1 (HTLV-1) infects 15-20 million people worldwide and is responsible of two major diseases : adult T cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). ATL in its acute/lymphoma phases is highly aggressive, resistant to current intensive chemotherapy and quickly mortal. HTLV-1 infection is characterized by viral latency in the large majority of infected cells and by the absence of viremia. These features are thought to be due to transcriptional repression of viral gene expression *in vivo*. Specific protein 1 (Sp1) is a transcription factor which binds to more than thousand different cellular promoters and regulates the expression of several genes involved in cell proliferation, apoptosis, and differentiation. Four Sp1 binding sites have been previously identified in the HTLV-1 promoter region. Since Sp1 is an important regulator of eukaryotic promoter transcriptional activity, we here analyzed the HTLV-1 Long Terminal Repeat (LTR) sequence for other potential Sp1 sites and studied the role of all the Sp1 sites in a nucleosomal context and in both HTLV-1 sense and antisense LTR promoter activity.
Results. *In silico* analysis of the HTLV-1 LTR promoter nucleotide sequence revealed the presence of two additional potential Sp1 binding sites within the R region. We demonstrated that the Sp1 and Sp3 transcription factors bound *in vitro* to these sites by EMSAs and supershift experiments. By competition assays, we compared the binding affinity for Sp1 of all six different HTLV-1 Sp1 binding sites and demonstrated, by chromatin immunprecipitation experiments, Sp1 recruitment *in vivo* to the newly identified Sp1 sites. Moreover, we demonstrated in the nucleosomal context of an episomal reporter vector that the Sp1 sites interfered with both the sense (from the 5'LTR) and antisense (from the 3'LTR) transcription. Interestingly, we showed that the two Sp1 binding sites located in the LTR R region exhibited together a repressor effect on the LTR sense transcriptional activity but had no effect on the LTR antisense activity.
Conclusion. Our results demonstrate the presence of two new functional Sp1 binding sites located in the R region of the HTLV-1 LTR, which act as negative *cis*-regulatory elements of sense transcription.

37.

The pseudorabies US3 protein interferes with presentation of the CD300a ligand phosphatidylserine and protects infected cells from NK mediated killing

Grauwet K (1), Cantoni C (2), Jacob T (1), De Pelsmaeker S (1), Claessen C (1), Parodi M (2), De Maria A (2), Moretta L (2), Vitale M (2), Favoreel H (1)

(1) Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium
(2) Department of Experimental Medicine, University of Genoa, Italy

Natural killer (NK) cells are key players in the innate response to viral infections. Killing of target cells by NK cells is regulated by a complex sum of signals received through activating/inhibiting receptors on the NK cell surface.
Here, we report that porcine SK cells infected with a porcine alphaherpesvirus pseudorabies virus (PRV) variant that lacks the viral kinase US3 (US3null PRV) are more susceptible to lysis by primary porcine NK cells than cells infected with wild type PRV (wt PRV), indicating a protective role of US3. Flow cytometric studies in primary porcine epithelial cells and SK cells show that the absence of US3 during PRV infection causes a reduced exposure of phosphatidylserine (PS) at the cell surface. Interestingly, PS is a known ligand for the inhibiting NK receptor CD300a in man (Borrego et al., 2012). Using the CD300a antibody IT144 in a redirected cytotoxicity assay using P815 cells and porcine NK cells, indicated that CD300a may also serve as an inhibitory receptor in porcine NK cells. To investigate whether the differences in PS exposure in cells infected with wt or US3null PRV translated in differences in CD300a binding efficiency, binding assay with recombinant CD300a were performed on primary porcine epithelial cells and SK cells. Cells infected with wt PRV showed a markedly increased binding of CD300a compared to mock-infected cells, while CD300a binding was not increased in cells infected with US3null PRV. We have shown before that PRV US3 triggers activation of group I p21 activated kinases (PAKs), central regulators of Rho GTPase signaling (Van den Broeke, 2009). To investigate a potential role of group I PAKs in our observations, PRV wt infected cells were treated with IPA-3, an inhibitor of group I PAKs. IPA-3 substantially decreased PS exposure on PRV wt infected cell surface, and almost completely abolished CD300a binding.
In conclusion, we demonstrate a novel function for the PRV US3 protein. Expression of US3 during infection suppresses NK cell-mediated lysis of infected cells, results in increased exposure of PS at the cell surface and in increased binding of the inhibitory NK receptor CD300a. The porcine CD300a is shown to be an inhibitory NK receptor in porcine NK cells, suggesting its involvement in the observed US3-mediated protection against NK cells.

38.

A comparative study of respiratory syncytial virus (RSV) infection of different murine macrophage cell lines reveals remarkable differences in susceptibility.

Heykers A., Leemans A., De Schryver M., Maes L., Cos P., Delputte PL

Laboratory for Microbiology, Parasitology and Hygiene (LMPH)
Department of Biomedical Sciences, University of Antwerp, Wilrijk

Contact: peter.delputte@uantwerpen.be

RSV is responsible for 64 million infections/year, with young children and elderly risking the development of severe bronchiolitis. Besides this severe acute pathology, RSV is also linked with chronic pulmonary problems, like asthma and recurrent wheezing. Because there is a link between the pathology and the host immune response, and because macrophages are, besides DC, prominent cells of the lung immune system, research groups have studied the role of macrophages during RSV infection. Interestingly, macrophages may not only play a role in RSV induced pathology, but also appear to be permissive for RSV infection in specific conditions. The obtained results however are often inconclusive and sometimes contradictory. A possible explanation is that diverse types of macrophages and macrophage cell lines were used in different studies. The aim of this study was therefore to evaluate the susceptibility of different types of macrophage cell lines for RSV infection.

The murine macrophage cell lines MH-S, RAW 264.7 and J774 were infected with the prototype RSV strain A2 and infectivity was compared to RSV infection of the epithelial type cell line HEP-2, which is traditionally used for *in vitro* RSV propagation and functional studies. Cells were inoculated with RSV and incubated at 37°C. To have a general idea of both RSV entry and infection, cells were respectively fixed at 2 and 24h post inoculation. Cells were subsequently permeabilized and RSV antigens were visualized by staining with a goat polyclonal anti-RSV serum followed by an AF 488-labelled donkey-anti-goat conjugate. As a control, mock-infected cells were used.

Fluorescent microscopy analysis showed that both MH-S (2%) and RAW 264.7 (0,4%) were infected, showing clear staining of RSV-antigens in the cytoplasm of the cells 24h p.i. This staining was clearly more intense compared to the cells that were stained at 2h p.i., indicating that new RSV antigens were synthesized and that virus was replicating. Surprisingly, J774 cells showed no clear positive signal of RSV antigens at 24h p.i.

Further analysis of MH-S and RAW 264.7 also revealed differences, since there are no RSV-antigens expressed on the surface of RAW 264.7 cells in contrast to MH-S cells. This suggests that RSV knows an abortive infection in RAW 264.7 cells, where the cell is indeed infected but where this does not result in the production of new virus particles. This assumption was explored with HEP-2 cells which were inoculated with supernatants of infected RAW 264.7 cells and MH-S cells, collected 24 and 72h p.i. The percentage of infected HEP-2 cells varied from 1,5 to 5% if it was inoculated with supernatants of MH-S from 24 or 72h p.i. This in contrast to HEP-2 cells inoculated with supernatants of RAW 264.7 cells, where the percentage of infected cells varied between 1,4 and 1,2%. In conclusion, we have shown that the RSV infection with the A2 strain varies among mouse macrophage cell lines.

39.

Natural killer and dendritic cells collaborate in the immune response induced by the vaccine against uterine cervical cancer

Inge Langers[1], Virginie Renoux[2], Anca Reschner[2], Antoine Touzé[3], Pierre Coursaget[3], Jacques Boniver[2], Joachim Koch[4], Philippe Delvenne[2], Nathalie Jacobs[1]

[1]Cellular and Molecular Immunology, GIGA-Research, University of Liège, Liège, Belgium.
[2]Experimental Pathology, GIGA-Research University of Liège, Liège, Belgium.
[3] Virologie et Immunologie Moléculaires, UMR 1282 INRA ISP, Université François Rabelais, Tours, France
[4] NK Cell Biology, Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany

Virus-like particles (VLP) of human papillomavirus (HPV) are used as a vaccine against HPV induced cancer and recently, we have shown that these VLP are able to activate natural killer (NK) cells. Since NK cells collaborate with dendritic cells (DC) to induce an immune response against viral infections and tumors, we studied the impact of this crosstalk in the context of HPV vaccination.
NK cells in the presence of HPV-VLP enhanced DC maturation as attested by an upregulation of CD86 and HLA-DR and an increased production of IL-12p70, but not of the immunosuppressive cytokine IL-10. Interestingly, we observed a decreased expression of NKp30 ligands on DC in the presence of NK cells and VLP. The activation was bi-directional. Indeed, in the presence of HPV-VLP, DC further activated NK cells by inducing the upregulation of cell surface activation markers (CD69 and HLA-DR), but not natural killer receptors such as NKp30, NKp44 and NKp46. The function of NK cells was also improved as shown by an increase in IFN-□ secretion and cytotoxic activity against HPV^+ cell line. This crosstalk between NK cells and DCs needed CD40 interaction and IL-12p70 secretion, whereas NKG2D was not implicated.
Our results provide insight into how VLP interact with innate immune cells and how NK cells and DC play a role in the immune response induced by this vaccine agent.

40.

Impact of caspase-1, -3, -7, RIPK3 and IL-1β/IL-18 deficiency on rabies virus infection and disease in mice

Kip E.[1,2,3], Suin V.[1], Nazé F.[1], Kalai M.[1], Beyaert R.[2,3], Vanden Berghe T.[2,3], Vandenabeele P.[2,3] and Van Gucht S.[1,4]

[1]National Reference Centre of TBEV and Rabies, Viral diseases, Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium
[2]Inflammation Research Center, VIB, Ghent, Belgium
[3]Ghent University, Department of Biomedical Molecular Biology, Ghent, Belgium
[4]Ghent University, Laboratory of Virology, Faculty of Veterinary Medicine, Ghent, Belgium

Rabies virus is a highly neurovirulent RNA virus, which causes 61000 deaths in humans each year. The clinical outcome of brain infection is most likely driven by an interplay between cell death, inflammation and neuronal dysfunction. However, the relative contribution of each of these processes is still unclear. We have therefore studied the impact on disease outcome of deficiency of different key proteins, involved in the signaling pathway of cell death: apoptosis (caspases-3, -7, -8, -9), pyroptosis (caspase-1, IL-1β/IL-18) and necroptosis (RIPK3).

In vitro, we found that rabies virus activates caspases-1, -3, -7, -8 and -9 in macrophages and triggers a strong apoptotic response. Moreover, knock-out of caspase-3, but not of caspase-1 or -7, partially inhibited virus-induced apoptosis in cultured bone-marrow derived macrophages. In wild type mice, intranasal inoculation with a lethal dose of neurovirulent rabies virus (CVS-11) leads to general brain infection and severe disease, with a median survival time of 8 days. Knock-out of caspase-1, -7 and IL-1β/IL-18 had no significant impact on disease progression. A short delay in disease progression was observed in mice deficient in caspase-3 or RIPK3. Eventually, all knock-out mice developed severe disease, with the same symptoms as in wild type mice, leading to euthanasia. High loads of rabies virus were detected in the brain of all knock-out mice, at similar levels as in wild type mice.

These results suggest that blocking of pathways specific for one type of cell death only leads to a limited (caspase-3, RIPK3) or no (caspase-1, IL-1β/IL-18, caspase-7) effect on rabies disease outcome. Often different pathways for cell death are redundant and simultaneous blocking of different pathways might be necessary to measure the impact of regulated cell death on rabies disease.

41.

Impact of MALT1 on rabies virus disease

Elodie Kip[1,2], Vanessa Suin.[1], Jens Staal[2], Michael Kalai[1], Rudi Beyaert[2] and Steven Van Gucht[1,3]

[1]National Reference Centre of TBEV and Rabies, Viral diseases, Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium.
[2]Unit of Molecular Signal Transduction in Inflammation, Inflammation Research Center, VIB and Ghent University, Department of Biomedical Molecular Biology, Ghent, Belgium.
[3]Ghent University, Laboratory of Virology, Faculty of Veterinary Medicine, Ghent, Belgium

Rabies virus is a highly neurovirulent RNA virus, which causes about 60000 human deaths each year. The clinical outcome of brain infection might be influenced by different host responses, such as, cell death, inflammation and/or lymphocyte activation. It is still unclear which host responses and signalling pathways contribute to disease development. We are therefore studying the impact of MALT1, a key protein involved in the signalling pathway of inflammation and lymphocyte activation upstream of NF-kB, on the outcome of rabies virus infection.

Cells or mice were infected with either the virulent CVS-11 strain (lethal in WT mice) or the attenuated ERA vaccine strain (non-lethal in WT mice). *In vitro*, MALT1 deficiency increased CVS and ERA production in MEF cells, as well as the percentage of infected cells. Correspondingly, a non-toxic concentration of mepazine (5µM), a MALT1 inhibitor, enhanced virus production in Jurkat T cells. On a cellular level MALT1 seems thus to have an antiviral role. *In vivo,* MALT1 KO significantly slowed down the development of rabies disease caused by the virulent CVS-11 strain. Upon euthanasia, viral loads in the brains were comparable between WT and KO mice. The delay of disease onset might be explained by a reduction in inflammation or lymphocyte/NK-driven neurotoxicity, which is supported by the lower IL-1β and IFN-ϒ levels in the brain. These results emphasize the importance of indirect (inflammatory/immunological) neurotoxic mechanisms on early rabies disease development, albeit all mice eventually die at a later time point of infection.

In contrast, the ERA viral strain, which is highly immunogenic and normally non-lethal, became lethal in MALT1 KO mice. Viral RNA loads were higher in the brains of KO mice than in those of WT mice, as well as the mRNA expression of IFN-β, TNF-alpha and RANTES. Possibly, MALT1 KO reduced the innate and/or lymphocyte-driven immune responses and thereby increased neuroinvasion with the ERA vaccine strain, which eventually led to disease and death, at a time point a few days later than with the virulent strain CVS-11 in KO mice. Treatment of WT mice with mepazine also increased the pathogenicity of ERA virus infection, albeit to a lower extent than with a full KO of MALT1.

42.

The US3 protein kinase of pseudorabies virus plays a role in viral mucosal invasion through the basement membrane

Jochen Lamote, Sarah Glorieux, Hans Nauwynck, Herman Favoreel

Faculty of Veterinary Sciences, Ghent University, Belgium

The US3 protein kinase of the alphaherpesvirus pseudorabies virus (PRV) causes a reorganisation of the host cell cytoskeleton and as such, is involved in efficient cell-to-cell spread in epithelial cell cultures (Favoreel et al. 2005. PNAS). US3 causes these cytoskeletal rearrangements by interfering with Rho-GTPase signaling. The US3 protein stimulates the Rac1 and Cdc42 signaling branch and suppresses the RhoA signaling branch (Van den Broeke et al. 2009. PNAS; Jacob et al. unpublished). Rho-GTPase signaling and its consequences on the organization of the cytoskeleton plays an important role in basement membrane (BM) transmigration of cells (Rowe & Weiss. 2008. Trends Cell Biol; Schoumacher et al. 2011. Eur J Cell Biol). Passage of the BM, which forms a barrier between epithelium and underlying lamina propria, forms a crucial step in the pathogenesis of alphaherpesviruses and may lead to further dissemination of the virus throughout the body via blood vessels and nerves.
Here, an ex vivo system of porcine nasal respiratory mucosa explants that is suitable to study PRV invasion through the BM (Glorieux et al. 2007. J Virol Methods), was used to study a potential role for US3 during BM passage of PRV. To this end, porcine nasal respiratory mucosa explants were inoculated with wild type (WT) PRV, US3-null PRV (which does not express US3 due to a premature stop codon) or US3-rescue PRV (with restored US3 mutation). These experiments showed that US3-null PRV was virtually unable to breach the BM, in contrast to WT and US3-rescue PRV. Interestingly, artificial suppression of RhoA signalling (using cell-permeable C3 transferase), to mimic the effect of US3 on Rho-GTPase signaling, increased passage of US3-null PRV through the BM (albeit not to WT PRV levels), whereas it did not affect BM passage of WT or US3-rescue PRV.
In conclusion, these data indicate that US3 plays an important role in PRV mucosal invasion through the basement membrane, at least in part via its interference with Rho-GTPase signaling.

43.

Production of Theiler virus trans encapsidated replicons

Lardinois Cécile, Gonzalez-Dunia Daniel, Michiels Thomas

Laboratoire de virologie – UCL
Avenue Hippocrate 74, bte B1.74.07
1200 Brussels

Previous reports suggest that Theiler's virus may traffic in the central nervous system via lysis-independant cell-to-cell transmission. In order to study this possibility, we produced trans encapsidated replicons derived from two strains of this virus (DA1 and GD7). In these replicons, the capsid coding region was replaced by either a GFP or a luciferase coding region.
To allow trans encapsidation of the RNA replicons, we generated a 293T cell line that constitutively expresses the TMEV capsid protein precursor via a lentiviral vector. We transfected the viral replicon RNA in these cells and we passaged them until a full cytopathic effect was observed.
We confirmed by immunofluorescence and FACS that the production of the capsid protein precursor in trans was a prerequisite for the propagation of the replicon in the cell population.
By successive centrifugation steps, we concentrated the encapsidated replicons and we obtained titers up to 107 infectious particles per ml.
To minimize the risk of contamination of our replicon stocks by recombinant infectious virus, we mutated CRE (a crucial RNA structure of the virus) in the capsid coding vector. We confirmed by plaque assay that no infectious virus contamined our replicon productions.
Finally, we tested our replicons on a primary neuron culture and confirmed that the GD7 derived replicon infects neurons, as the fully infectious virus does.
In conclusion, we generated a very interesting tool that will allow to dissect the mechanisms of viral cell to cell propagation in neurons *in vitro* and in different *in vivo* models.

44.

Murid herpesvirus 4 ORF63 is involved in the translocation of incoming capsids to the nucleus

Muhammad Bilal Latif, Bénédicte Machiels, Alain Vanderplasschen. Laurent Gillet

Laboratory of Immunology-Vaccinology, FARAH, University of Liège, Belgium

Herpesvirus virions consist of four morphologically distinct structures: DNA core, capsid, tegument and envelope. The tegument consists of about 20 different viral proteins and plays diverse roles in the virus lifecycle, including: capsid transport during entry and egress; regulation of transcription and translation; viral DNA replication; viral assembly and immune modulation. Most of these functions have been defined in alphaherpesviruses. Interestingly, a recent study identified a potential role in immune evasion for the tegument protein encoded by ORF63 of gammaherpesviruses. However, this study did not involve the construction of ORF63 knockout strain and the significance of these results in the viral lifecycle remains unknown. In this project, we wanted therefore to define the functional importance of ORF63 expression during Murid Herpesvirus 4 (MuHV-4) infection *in vitro* and *in vivo*. We showed that a lack of the ORF63 in MuHV-4 was associated with a severe viral growth deficit both *in vitro* and *in vivo*. The latter deficit was mainly associated with a defect during the viral lytic cycle in the lung but did not appear to be due to a reduced ability to establish latency. On a functional point of view, inhibition of caspase-1 or NLRP3 inflammasome did not restore the growth of the ORF63 deficient mutant suggesting that the observed deficit was not associated with the immune evasion mechanism identified previously. Moreover, this growth deficit was also not associated with a defect in virion egress from the infected cells. In contrast, it appeared that the entry process of MuHV-4 virions was deeply affected by the absence of ORF63. Indeed, MuHV-4 ORF63 deficient mutants failed to address most of their capsids to the nucleus, suggesting that ORF63 plays a role in capsid movement along the microtubule network. Altogether, this study provides new insights into the mechanisms used by gammaherpesvirus capsids to reach the nucleus during entry.

45.

Equine herpesvirus type 1 (EHV-1) replication is delayed in CD172a$^+$ monocytic cells and controlled by histone deacetylases

Kathlyn Laval, Herman W. Favoreel, Hans J. Nauwynck

Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medecine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

Abstract

Equine herpesvirus type 1 (EHV-1) replicates in the epithelial cells of the upper respiratory tract and disseminates through the body via a cell-associated viremia in monocytic cells, despite the presence of neutralizing antibodies. However, the mechanism by which EHV-1 hijacks immune cells and uses them as 'Trojan horses' in order to disseminate inside its host is still unclear. Here, we hypothesize that EHV-1 delays its replication in monocytic cells in order to avoid recognition by the immune system. We compared replication kinetics *in vitro* of EHV-1 in RK-13, a cell line fully susceptible to EHV-1 infection, and primary horse cells from the myeloid lineage (CD172a$^+$). We found that EHV-1 replication was restricted to 4% of CD172a$^+$ compared to 100% in RK-13. In susceptible CD172a$^+$, the expression of immediate-early (IEP) and early (EICP22) proteins was delayed in the cell nuclei by 2-3 hpi compared to RK-13, and the formation of replicative compartments by 15 hpi. The viral production in CD172a$^+$ was significantly lower (from $10^{1.7 \text{ to } 3.1}$ $TCID_{50}/10^5$ inoculated cells) than in RK-13 (from $10^{5 \text{ to } 5.7}$ $TCID_{50}/10^5$ inoculated cells). Less than 0.02% of inoculated CD172a$^+$ produced and transmitted infectious virus to neighbor cells. Pretreatment of CD172a$^+$ with inhibitors of HDAC activity increased and accelerated viral protein expression at very early time of infection and induced productive infection in CD172a$^+$. Our results demonstrated that the restriction and delay of EHV-1 replication in CD172a$^+$ is part of an immune evasive strategy and involves silencing of EHV-1 gene expression associated with histone deacetylases.

46.

Antibody-induced internalization of RSV F protein expressed on the surface of infected cells and cells expressing a recombinant protein

Leemans A., De Schryver M., Heykers A., Maes L., Cos P., Delputte PL

Laboratory of Microbiology, Parasitology and Hygiene (LMPH) Department of Biomedical Sciences, University of Antwerp, Wilrijk

Similar to several other viruses, RSV expresses viral proteins on the surface of infected cells, which can be detected and targeted by RSV-specific antibodies. Previous studies have shown that upon binding of polyclonal RSV-specific antibodies to RSV antigens expressed on the surface of infected HEp-2 cells, internalization may occur of these RSV antigen-antibody complexes, presumably by a clathrin-dependent mechanism.
For further elucidation of the viral protein(s) involved in these processes and confirmation of the mechanism of internalization, cells transfected with the RSV F protein were used, instead of infected cells, to study antibody-induced internalization. Transfected cells were incubated with polyclonal RSV-specific antibodies at 37°C during different times (0 min up to 120 min), followed by fixation, permeabilization and staining with secondary labeled antibodies. Analysis of the cells was performed by confocal fluorescence microscopy. Addition of polyclonal RSV-specific antibodies resulted in a clear uptake of viral protein-antibody complexes in a time-dependent manner. This observation indicates that at least the RSV F protein is involved in antibody-induced internalization of RSV proteins at the surface of infected cells. The process was rapid and stagnated between 60 and 90 minutes after the addition of antibodies. By using RSV-infected cells and F-specific monoclonal antibodies, the involvement of the RSV F protein in this internalization process was further confirmed in the context of RSV infection. Upon internalization, complexes of viral proteins and antibodies were transported towards a perinuclear region resembling the lysosomal compartment. Also a clear reduction in surface expression was observed and confirmed by flow cytometric analysis of RSV infected cells. Experiments with specific inhibitors of different endocytic pathways and co-transfection with dominant negative proteins indicate that this process of antibody-induced internalization of the RSV F protein is clathrin dependent. Currently, experiments are ongoing to analyze whether internalization of the viral protein-antibody complexes interferes with antibody-dependent complement-mediated lysis.

47.

Bovine herpesvirus 4 modulates its beta-1,6-N-acetylglucosaminyltransferase activity through alternative splicing

Céline Lété [1], Nicolas Markine-Goriaynoff [1], Bénédicte Machiels [1], Poh-Choo Pang[3], Kevin Canis[3], Minoru Fukuda[2], Anne Dell[3], Stuart M Haslam[3], Alain Vanderplasschen[1], Laurent Gillet[1]

[1]Immunology-Vaccinology, Department of Infectious and Parasitic Diseases (B43b), FARAH, Ulg.
[2]Division of Molecular Biosciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, UK
[3]The Burnham Institute, 10901 North Torrey Pines Rd, La Jolla, CA 92037, USA

The Bo17 gene of Bovine herpesvirus 4 (BoHV-4) is the only virus gene known to date that encodes a homologue of the cellular core 2 β-1,6-*N*-acetylglucosaminyltransferase-mucin type (C2GnT-M). The nucleotide sequence of the Bo17 gene has 95% identity with the cellular sequence from which it was acquired. However, by opposition to what is observed for the cellular gene, we show in this study that two different transcripts are encoded by the Bo17 gene. The first one corresponds to the entire coding sequence of the Bo17 gene and, surprisingly, the second results from the splicing of a 138 bp intron. Analysis of different homologous sequences revealed that, compared to cellular sequences, only Bo17 gene presents the consensus sites for splicing and that these sites are conserved in all the BoHV-4 strains identified to date. This splicing does not change the reading frame of the protein and antibodies generated against Bo17 C-terminus showed that the two forms of Bo17 are expressed in BoHV-4 infected cells. However, by using an *in vitro* assay, we showed that the spliced form of Bo17 is not anymore active. In order to test the role of these two forms in the viral lifecycle, recombinant strains expressing only the long or the short form of Bo17 were constructed. Interestingly, we showed that BoHV-4 could use alternative splicing in order to modulate the cellular C2GnT-M activity. We postulate that the relative abundance of active/inactive forms of pBo17 in Golgi oligomeric complexes may define the level of C2GnT-M activity in the cell. This new regulatory mechanism could have implication in viral immune evasion but also more generally in cellular biology.

48.

Reduced susceptibility of CD4$^+$ T-cells from elite controllers to HIV-1 infection reveals a block at a step between reverse transcription and integration

Paradise Madlala[1,2], Rik Gijsbers[1], Sofie Vets[1], Erik Van Wijngaerden[3], Rik Schrijvers[1,3], and Zeger Debyser[1]

[1] KU Leuven, University of Leuven, Laboratory for Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, Leuven, Belgium
[2] HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa
[3] KU Leuven, University Hospitals, Internal Medicine Leuven, Belgium

Abstract

Background: Elite controllers (EC) are a rare group of human immunodeficiency virus type 1 (HIV-1) infected individuals who naturally control HIV-1 replication to levels below limit of detection without antiretroviral therapy (ART) and who rarely progress to AIDS. Understanding of how EC control viral replication could provide valuable clues to guide research on vaccine development and virus eradication. Although previous studies have implicated defective viruses, adaptive immune response, restriction factors, and more recently possible cellular mechanisms as reasons of reduced HIV-1 replication in EC, none of the explanations has been exhaustive. In the present study, we sought to identify the step at which HIV-1 replication is blocked in some EC and to study the role of cellular cofactors in modulating HIV-1 replication.

Methods: We investigated HIV replication in peripheral blood mononuclear cells (PBMC) obtained from two discordant couples followed up at the Gasthuisberg University Hospital in Leuven. In each discordant couple one partner is an EC and the other an HIV-1 progressor (PR). PMBCs from healthy volunteers were used as controls (HC). CD4$^+$ T cells were activated with IL-2 and anti-CD3/CD8 bispecific antibodies and infected on day 5 after stimulation with R5-tropic HIV-1 strain 91US056 (MOI 0.001). HIV-1 infection was monitored by p24 ELISA. In addition to a multiple round virus, activated CD4$^+$ T-cells were also infected with yellow fluorescent protein-encoding (YFP-encoding) single-round HIV-1 virus (NL4.3). The different viral DNA species formed during HIV infection were determined by quantitative PCR (qPCR).

Results: CD4$^+$ T-cells from EC proved 8-10 fold less susceptible to multiple round HIV-1 (BAL) infection when compared to CD4$^+$ T cells derived from PR or HC. This result was corroborated by results obtained with the YFP-encoding single-round virus. The 2-fold reduction in infection of EC CD4$^+$ T-cells with YFP-encoding, VSV-G–pseudotyped HIV-1 vector compared to cells from HC or PR, suggests a block during the early steps of HIV-1 replication in EC. Quantification of early steps of replication demonstrated reduced numbers of reverse transcripts and proviral DNA integrants in CD4$^+$ T-cells form EC compared to PR and HC, demonstrating a block between reverse transcription and integration. Next we investigated whether reduced integration levels in EC were associated with an altered integration site distribution profile. However, no differences were observed when comparing cells from EC and PR. Lastly we investigated the expression levels of cellular cofactors such as LEDGF/p75, transportin-SR2 and p21 in EC samples. No differences in mRNA expression levels of cellular cofactors were observed between EC and PR.

Conclusion: We were able to phenocopy the EC phenotypes observed in one partner of two discordant HIV-1 infected couples by infecting their CD4$^+$ T-cells with lab strains of HIV-1. The reduced permissiveness to HIV-1 infection in the CD4$^+$ T-cells from both ECs is mediated by a block in HIV-1 replication at a step between reverse transcription and integration.

49.

Development of a novel St. Louis encephalitis mice model: Insight into disease pathogenesis

Rafael Elias Marques*, Rebeca Rocha, Juliana del Sarto, Mauro Martins Teixeira

*Rega Institute, KU Leuven, Belgium
Immunopharmacology Lab, Universidade Federal de Minas Gerais, Brazil

St. Louis encephalitis virus (SLEV) is a causative agent of human and veterinary encephalitis in the western hemisphere. SLEV is included in the *Flavivirus* genus together with important human pathogens such as *Dengue virus* and *West Nile virus*. At the onset of encephalitis, mortality rates can reach 30% and the possibility of neurological sequelae is significant. Although St. Louis encephalitis pathogenesis is poorly understood, misguided or excessive inflammatory responses in the central nervous system are thought to contribute to disease development. In order to investigate the immune/ inflammatory responses to SLEV *in vivo*, we developed an experimental model of St. Louis encephalitis in mice. Intracranial inoculation of our SLEV strain (isolated from a Brazilian symptomatic patient) in adult wild-type C57BL/6 mice caused death in approximately 7 days, in an inoculum-dependent fashion. Viral load assessment by plaque assay and RT-qPCR indicated that SLEV replicates extensively in the brain, yet SLEV could not be detected in the periphery. Interestingly, viral load in brain reach peak values at 7 days post-infection (p.i.), concomitant with mice death. We observed that SLEV replication in mice brains induced pro-inflammatory cytokine and chemokine production, markedly IFN-γ, CXCL1 and CCL5, measured by ELISA. Flow cytometry data showed that SLEV infection led to an intense leukocyte recruitment to the brain, composed mainly by neutrophils and lymphocytes and corroborating chemokine production. Excessive cytokine production and leukocyte recruitment to the infected brain is consistent with encephalitis and is associated to brain tissue damage, which is evident at day 7 p.i. Thus, we considered that the peak of disease in this model is at 7 days p.i., when infection, inflammation and tissue damage lead to mice death. Finally, SLEV infection in mice caused behavioral alterations comparable to neurological alterations observed in human encephalitis, such as paralysis and reduction in neuropsychological scores, assessed with the SHIRPA test. In summary, our experimental SLEV infection model resembles important aspects of human St. Louis encephalitis and could contribute to the understanding of this disease and others caused by related flaviviruses. More important, we conclude that inflammation is a major component in experimental St Louis encephalitis and likely contributes to disease pathogenesis rather than protection.

50.

Experimental infection of sheep with Schmallenberg virus at days 45 and 60 of pregnancy.

Ludovic Martinelle[1#], Antoine Poskin[1,2#], Fabiana Dal Pozzo[1], Brigitte Cay[2], Nick De Regge[2] and Claude Saegerman[1]
# both authors contributed equally to the work

[1.] Unit of Research on Epidemiology and Risks Analysis, FARAH, ULg, Liège.
[2.] Veterinary and Agrochemical Research Centre, Virology Department, Uccle, Brussels.

Corresponding author: claude.saegerman@ulg.ac.be

In summer 2011, a new unspecific clinical entity has been first described in adult cattle in Germany, later attributed to a novel *Orthobunyavirus*, named Schmallenberg virus (SBV). SBV is associated with febrile disease, milk drop and diarrhoea in adult cattle, and with reproductive disorders, including teratogenic effects, abortions and stillbirth in ruminants' offspring. Characteristics of placental crossing are currently poorly understood and gestation susceptible periods that lead to congenital malformations need to be clarified in host species. Therefore, we implemented an experimental infection of ewes, inoculated with SBV at 45 or 60 days of pregnancy.
"Mourerous" breed ewes, about 1-year old, were separated in three groups: eight and nine ewes were subcutaneously inoculated with 1 ml of SBV infectious serum at 45 and 60 days of pregnancy, respectively (G45 and G60). Six other ewes were inoculated with sterile PBS and constituted a control group (GC). All inoculated ewes showed RNAemia consistent with previously published studies, they seroconverted and no clinical signs were reported. Lambs were born at term via C-section, and right after birth they were blood sampled ad clinically examined. Then both lambs and ewes were euthanatized and necropsied.
No lambs showed any malformation suggestive of SBV infection and none of them had RNAemia or anti-SBV antibodies prior to colostrum uptake. However viral RNA was found in umbilical cord and placenta of two different ewes in G45. In G60, viral RNA was found in the placenta of 4 ewes, the cotyledon of 3 ewes and the umbilical cord of 3 ewes, along with the prescapular lymph node and cartilage of one lamb and the brainstem of another one.
Overall, no teratogenic effect could be reproduce; however the highest detection rate of viral RNA in G60 suggests a time dependency for successful tansplacental transmission and persistence of the virus until birth.

51.

The Cyprinid herpesvirus 3 – carp model: a unique model to test the roles in anti-viral innate immunity of Zalpha domain proteins detecting unusual nucleic acid conformations

Morvan L., Rakus K., Boutier M., Vanderplasschen A.

Laboratory of Immunology-Vaccinology, University of Liège.

The innate immune system relies on a large number of molecules that act as sensors of nucleic acid. Nucleic acids are detected based on their structure, their subcellular localization or even their sequence. According to these criteria, their detection can be interpreted by the innate immune system as the presence of a pathogen (pathogen associated molecular pattern, PAMP) or host cell damage (damage associated molecular pattern, DAMP). Zalpha domains are 66 aa long domains which bind to left-handed dsDNA (Z-DNA) or left-handed dsRNA (Z-RNA). The description of Zalpha domains in proteins belonging to the host innate immune system but also in viral proteins suggests that even the conformation of the nucleic acid could be exploited by the innate immune system as a PAMP or DAMP. The present project aims to test this interesting and original hypothesis both *in vitro* and *in vivo* taking advantage of the Cyprinid herpesvirus 3 (CyHV-3) -Carp model. Carp, like all Cyprinid fish species, expresses PKZ, a paralogue of the dsRNA-dependent protein kinase (PKR) expressed by all vertebrates. While PKR possesses dsRNA binding domains, PKZ has Zalpha binding domains. Both proteins once activated by binding to the appropriate nucleic acids (dsRNA and Z-DNA/RNA, respectively) phosphorylate eIF-2α thereby blocking protein synthesis. This leads to accumulation of preinitiation complexes containing 40S subunits and later, to the organization of these with mRNPs into stress granules. Very recently, it was demonstrated that ORF112 of CyHV-3 encodes a Zalpha domain protein (pORF112) over competing the binding of PKZ to Z-DNA. Experiments conducted on this topic strongly suggest that the ORF112 gene is essential for replication of the virus in cell culture. All together, these observations suggest that PKZ could play a key role in innate anti-viral immunity that needs to be evaded by CyHV-3 ORF112 to allow efficient viral replication. As both PKZ and pORF112 possess Zalpha binding domains, the CyHV-3/carp model provides a unique opportunity to test the hypothesis that detection of unusual conformation of nucleic acid could be sensed by Zalpha domain proteins as PAMP and DAMP. The physiological relevance of this original hypothesis will be tested both *in vitro* and *in vivo*.

52.

Viral semaphorin inhibits dendritic cell phagocytosis and migration but is not essential for γ-herpesvirus-induced lymphoproliferation in malignant catarrhal fever

Françoise Myster[a], Leonor Palmeira[a,b], Océane Sorel[a], Edwin DePauw[c], Isabelle Schwartz-Cornil[d], Alain Vanderplasschen[a], Benjamin G. Dewals[a]

[a]Fundamental and Applied Research in Animals and Health (FARAH), Immunology-Vaccinology, Faculty of Veterinary Medicine (B43b); [b]GIGA-Research, Unit of Animal Genomics, Faculty of Veterinary Medicine (B43); [c]Mass Spectrometry Laboratory, Department of Chemistry, University of Liège, B-4000 Liège, Belgium; [d]Virologie et Immunologie Moléculaires, UR892 INRA, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France.

Although semaphorins belong to a protein family first identified as axonal guidance factor, evidences show now that semaphorins are involved in a wide variety of biological processes in many different organ systems. Semaphorins have been implicated in various phases of the immune response, from initiation to terminal inflammatory processes. Viral semaphorins are semaphorin 7A (sema7A) mimics found in pox- and herpesviruses. Among herpesviruses, semaphorins are encoded by γ-herpesviruses of the Macavirus genus only. Alcelaphine herpesvirus 1 (AlHV-1) is a Macavirus that persistently infects wildebeest asymptomatically but induces malignant catarrhal fever (MCF) when transmitted to several species of susceptible ruminants and the rabbit model. MCF is caused by the activation/proliferation of latently infected T lymphocytes. Viral semaphorins have been suggested to mediate immune evasion mechanisms and/or directly alter host T cell function. Here we studied AlHV-sema, the viral semaphorin encoded by the A3 gene of AlHV-1. Phylogenetic analyses revealed independent acquisition of pox- and herpesvirus semaphorins, suggesting that these proteins might have distinct functions. AlHV-sema showed a predicted 3-D structure very similar to sema7A and conserved key residues in sema7A-plexinC1 interaction. Expression analyses revealed that AlHV-sema is a secreted 93 kDa glycoprotein expressed as a homodimer during the early phase of virus replication. Purified AlHV-sema was able to bind to fibroblasts and dendritic cells and induce F-actin condensation and cell retraction. It acts through the viral semaphorin receptor plexinC1 leading to a Rho/cofilin dependent mechanism. Cytoskeleton rearrangement was further associated with inhibition of phagocytosis by dendritic cells and migration to the draining lymph node. Then, we used recombinant viruses and demonstrated that the lack of A3 did not significantly affect virus growth *in vitro* and did not impair MCF induction and associated lesions. In conclusion, AlHV-sema has immune evasion functions through mechanisms similar to poxvirus semaphorin and is not directly involved in host T cell activation during MCF.

53.

Are the multiple functions of Theiler's virus Leader (L) protein coupled?

PEETERS Michael

VIRO (UCL, 74 Avenue Hippocrate, 1200 Brussels) – MICHIELS Thomas

Cardioviruses produce a very short leader (L) protein. Four 'domains' have been recognized in this protein: a N-terminal Zn finger domain, an acidic domain, a serine/threonine-rich domain and a C-terminal domain called Theilodomain conserved in *Theilovirus* L proteins but absent from the related L protein of EMCV. L proteins interfere with numerous cell functions (Ricour et al., 2009 ; Borghese and Michiels, 2011 ; Porter et al., 2010): interferon and chemokine production, apoptosis induction, stress granules formation, protein synthesis, MAP kinase activation and nucleo-cytoplasmic trafficking. We recently observed that L interacts with cellular kinases of the RSK family. This work aimed at defining whether the multiple activities of L derive from a single master activity of L (i.e. interference with RSK function) or if its various activities are uncoupled.

To this end, we mapped L residues that are essential for host cell protein shut-off and for RSK interaction. Next, we analyzed whether theses residues are also important for other L functions such as the perturbation of nucleocytoplasmic trafficking and nucleoporins hyperphosphorylation.

L residues involved in RSK binding were mapped by co-immunoprecipitation. In addition to the Zn finger, we identified a motif within the acidic domain, which is critical for RSK binding. The Theilodomain was not required for RSK binding.
Mutations that affected the most RSK binding also affected nucleo-cytoplasmic trafficking, hyperphosphorylation of nucleoporins and protein synthesis shut-off, suggesting that RSK binding may be related to the other L activities.
Interestingly, the Theilodomain, which was critical for host protein shut-off and nucleo-cytoplasmic trafficking was not required for RSK binding.

This suggests a model where the core of L would recruit RSK and the C-terminal domain would recruit kinase targets.
The leader protein might thereby modulate the target spectrum of RSK kinases.

Ref.
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54.

Identification of cyprinid herpesvirus 3 (CyHV-3) envelope transmembrane proteins that are essential to viral growth *in vitro*

Ma. Michelle D. Peñaranda[1], Vancsok C.[1], Jazowiecka-Rakus J.[1], Stalin Raj V.[1, 2], Vanderplasschen A.[1]

[1]. Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, 4000, Liège, Belgium
[2]. Present Affiliation: Erasmus MC, Department of Virology, Zuid Holland, Netherlands

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is the aetiological agent of an emerging and lethal disease in koi (*Cyprinus carpio koi*) and common carp (*C. carpio carpio*). Its genome consists of a linear, double-stranded DNA of about 295 kb encoding 155 open reading frames. Viral envelope proteins expressed on the surface of the virion play key role in viral entry, egress and pathogenesis. The present study aims to investigate which CyHV-3 envelope proteins are essential to viral growth *in vitro*. To reach this goal, a two steps approach was developed. First, two-dimensional liquid chromatography tandem mass spectrometry (2D-LC MS/MS) was performed on purified CyHV-3 virions to identify structural proteins. Based on bioinformatics analyses 16 transmembrane proteins were identified among structural proteins. Second, for each gene encoding these 16 transmembrane proteins, we produced a recombinant deleted strain using bacterial artificial chromosome (BAC) cloning and prokaryotic recombination technology; and subsequently, tested the ability of the recombinant virus to replicate in common carp brain (CCB) cells. Deletion of ORF32, ORF59, ORF81, ORF99, ORF115, and ORF131 impaired viral growth *in vitro*. Reversion of the deletion restored the ability of the virus to replicate. In contrast, deletion of CyHV-3 genes belonging to the ORF25 family (ORF25, ORF65, ORF148, and ORF149), ORF108, ORF132, and ORF136 were not essential to viral growth *in vitro*. Investigation on three other CyHV-3 envelope proteins, ORF64, ORF83, and ORF106 is currently on-going. Preliminary results suggest that ORF83 and ORF106 are also essential for *in vitro* replication while ORF64 is not.

55.

Pseudorabies virus gE causes ERK1/2 activation in primary porcine T lymphocytes and subsequent cell aggregation and migration

M. Pontes, B. Devriendt, H. W. Favoreel

Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium

Alphaherpesviruses are the largest subfamily of the Herpesviruses. This subfamily contains closely related pathogens, including the herpes simplex virus 1 and 2 (HSV -1 and HSV-2), and varicella zoster virus (VZV) in man. Another member of the alphaherpesvirus subfamily is the porcine pseudorabies virus (PRV) that is often used as a model to study general features of alphaherpesvirus biology (Pomeranz, 2005, MMBR).
We previously demonstrated that Pseudorabies virus (PRV) activates the MAP kinase Erk 1/2 in Jurkat T-cells. Activation of Erk 1/2 was dependent on the extracellular domain of viral glycoprotein gE, revealing a new role for gE during alphaherpesvirus infection.
To corroborate our findings in a porcine system, which is the natural host of PRV, we used primary T lymphocytes isolated from porcine peripheral blood mononuclear cells. The isolated T lymphocytes were co-cultured with gE-expressing porcine epithelial cells. In line with our previous observations in Jurkat T-cells, gE caused a rapid and transient activation of Erk 1/2 in primary T lymphocytes.
In a next step, we wanted to address potential biological consequences of gE-mediated Erk 1/2 activation in T lymphocytes. One of the consequences of Erk 1/2 signaling in T lymphocytes described in literature is T lymphocyte aggregation, which is indicative for T cell activation (Layseca-Espinosa et al., 2003, JLB). At 24h post inoculation (hpi), wild type (WT) PRV caused a substantial increase in primary porcine T lymphocyte aggregation, leading to the formation of large cell aggregates when compared to mock-infected cells. An isogenic gEnull PRV variant also induced some cell aggregation, this was significantly less pronounced compared to wild type PRV. Importantly, addition of U0126, an inhibitor of Erk 1/2 phosphorylation, drastically reduced WT PRV-induced T lymphocyte aggregation levels to mock levels. Cellular aggregation often correlates with cell migration (Layseca-Espinosa et al., 2003; Jevnikar et al., 2008). Using a Transwell migration system, we found that at 24 hpi, WT PRV led to a significantly increased migration of primary porcine T lymphocytes when compared to mock-infected cells. The gEnull PRV variant caused a less pronounced migration, whilst U0126 almost abrogated WT PRV-induced migration. Collection of migrated T lymphocytes and co-culture of these cells with porcine ST cells resulted in obvious virus plaques in ST cells, indicating that migrating T lymphocytes are able to transmit PRV to other susceptible cells.
Taken together, our results indicate a potential new role for gE in viral spread, where gE-mediated ERK 1/2 activation triggers PRV-carrying T lymphocytes to migrate and possibly infect other cells susceptible to PRV replication.

56.

Cyprinid herpesvirus 3 encodes a soluble homologue of the mammalian TNFRSF14 receptor: roles in the biology of the infection *in vitro* and *in vivo*

Jazowiecka-Rakus J.[1], Rakus K.[1], Boutier M.[1], Reschner A.[1], Ronsmans M.[1], Vanderplasschen A.[1]

[1]Immunology-Vaccinology, University of Liège

Corresponding author: jrakus@ulg.ac.be

TNFR (tumour necrosis factor receptor) homologues were reported in different viruses including herpesviruses and poxviruses and were shown to play a role in immune evasion mechanisms. Cyprinid herpesvirus 3 (CyHV-3) causes a lethal disease in common and koi carp. CyHV-3 ORF4 encodes a soluble homologue of the mammalian TNFRSF14 membrane receptor (also called HVEM). Proteomic analysis of CyHV-3 infected cells demonstrated the absence of ORF4 expression product in the CyHV-3 secretome but suggested its presence in CyHV-3 virions.

To investigate the roles of ORF4 in the biology of CyHV-3 infection, two recombinant strains deleted for ORF4 were produced using BAC cloning technologies. Restriction profile, Southern blot and full genome sequencing confirmed the correct molecular structure of these recombinant strains. *In vitro*, the two deleted strains replicated comparably to the control strain expressing ORF4. *In vivo* studies showed a mild difference in kinetics of the infection between fish infected with ORF4 deleted strains and the control strain. The development of the disease (clinical signs) and the peak of virus load (measured by real-time TaqMan PCR in gills and heart) were delayed in fish infected with ORF4 deleted strains. Nevertheless, the cumulative mortality rates were comparable for the three viruses tested.

The results of the present study demonstrate that CyHV-3 ORF4 could play a role in pathogenesis at the early stages of the disease. Additional research is required to study the interaction between CyHV-3 ORF4 and the carp immune system.

57.

TNF-α induces behavioral fever in common carp (*Cyprinus carpio*).

Rakus K.[1], Ronsmans M.[1], Forlenza M.[2], Boutier M.[1], Jazowiecka-Rakus J.[1], Penaranda M.[1], Farnir F.[3], Becco C.[4], Piazzon C[2]., Michiels T[5]., Wiegertjes G.[2], Vanderplasschen A.[1]

[1.]Immunology-Vaccinology, FARAH, ULg.
[2.]Cell Biology and Immunology, Wageningen University, The Netherlands.
[3.]Biostatistics and Bioinformatics applied to veterinary sciences, FARAH, Ulg.
[4.]Institute of Physics, Ulg.
[5.]de Duve Institute, UCL.

Fever in response to infectious or inflammatory agents is an ancestral mechanism of innate immunity existing in all vertebrates including fish. Since fish cannot rely on endogenous thermoregulatory mechanisms to control their body temperature, they mainly use behavioral thermoregulation. This process is based on active selection of a given temperature to optimize the metabolic processes in heterogeneous thermal habitats. Also, fever in fish is based on a behavior modification stimulating the individual to reside in a warmer environment, hence is named behavioral fever. Cyprinid herpesvirus 3 (CyHV-3) is the causative agent of a lethal disease in common and koi carp. The disease occurred mainly when water temperature is between 18°C and 28 °C.

To study the mechanisms of behavioral fever in carp during CyHV-3 infection, we developed a multi-chamber aquaria with temperature gradient (24 ^{o}C - 28 ^{o}C - 34 ^{o}C) and informatics system to monitor the position of the fish. The results of our study demonstrated that: **1) CyHV-3 infection induces behavioral fever in carp.** Infected fish migrated to the compartment with the highest temperature (34 ^{o}C) and stayed there until controlling the disease. **2) Behavioural fever induced by CyHV-3 is salutary.** Migration of infected fish to 34 ^{o}C resulted in the absence of lethality while mortality rates of 100% and 80% were observed when the fish were maintained in control tanks at 24 °C and 28 °C, respectively. **3) CyHV-3 ORF12 encodes a soluble TNF-α receptor that delays the onset of behavioural fever induced during the disease.** *In vitro* studies demonstrated that CyHV-3 ORF12 encodes a soluble abundantly secreted protein able to bind and to neutralize both carp TNF-α1 and TNF-α2. CyHV-3 recombinant strain deleted for ORF12 induced behavioural fever earlier than the control revertant strain. **4) Blocking of TNF-α inhibited behavioural fever.** Anti-carp TNF-α antibodies administrated passively blocked migration of infected fish to the 34 ^{o}C compartment. **5) TNF-α1 is sufficient to induce behavioural fever in a dose dependent manner.** Fish injected with expression plasmids encoding TNF-α1 migrated to 34 ^{o}C in contrast to fish injected with plasmid encoding TNF-α2 or no transgene.

58.

The replication characteristics of infectious laryngotracheitis virus (ILTV) in the respiratory and conjunctival mucosa

Vishwanatha R.A.P. Reddy[1], Lennert Steukers[1], Yewei Li[1], Walter Fuchs[2], Alain Vanderplasschen[3] and Hans J. Nauwynck[1*]

[1]Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[2]Institute of Molecular Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Boddenblick 5A, 17493 Greifswald - Insel Reims, Germany
[3]Laboratory of Immunology-Vaccinology (B43b), Department of infectious and parasitic diseases, Faculty of Veterinary Medicine, University of Liege, B-4000 Liege, Belgium

Abstract

Avian infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus of poultry that is spread worldwide. ILTV enters its host via respiratory tract and eyes. Although the virus is known for a long time, the replication characteristics of ILTV in the respiratory and conjunctival mucosa are still poorly studied. To study the replication characteristics of ILTV in chicken tracheal and conjunctival mucosa, two *in vitro* explant models were developed. Light microscopy and a fluorescent terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining were used to evaluate the viability of mucosal explants. The mucosal explants were viable up to the end of the experiment at 96h of cultivation. The tracheal and conjunctival mucosal explants were inoculated with ILTV and collected at 0, 24, 48 and 72h post inoculation (pi). ILTV spread in a plaquewise manner in both mucosae. A reproducible quantitative analysis of this mucosal spread was evaluated by measuring plaque numbers, plaque latitude and invasion depth underneath the basement membrane (BM). No major differences in the plaque numbers were observed over time. Plaque latitude progressively increased over time up to 70.4 ± 12.9 μm in trachea and 97.8 ± 9.5 μm in conjunctiva at 72h pi. The virus had difficulties to cross the BM. Only from 48h pi, BM crossing was found and at 72h pi it was observed in 56% (trachea) and 74% (conjunctiva) of the plaques. Viability analysis of infected explants indicated that ILTV blocks apoptosis in infected cells of both mucosae but activates apoptosis in bystander cells.

59.

ORF9 tegument protein: a new player in Varicella-zoster virus nuclear egress

Laura Riva,[1$] Marc Thiry,[2] Marielle Lebrun,[1] Laurent L'homme,[3] Jacques Piette,[1 3] Catherine Sadzot-Delvaux [1] *

University of Liege, GIGA-Infection Immunity and Inflammation, Laboratory of Virology and Immunology, Liege, Belgium [1]
University of Liege, GIGA-Neurosciences, Laboratory of Cellular and Tissular Biology, Liege, Belgium [2]
University of Liege, GIGA-Signal Transduction, Laboratory of Virology and Immunology, Liege, Belgium [3]

ABSTRACT
ORF9 is the most transcribed Varicella-zoster virus (VZV) gene during infection and the protein encoded by this ORF is essential for VZV replication. ORF9p has been previously observed to be present in the trans-Golgi network and to play a role in secondary envelopment. In this work we deleted the ORF9p acidic cluster, which overlaps the previously identified ORF47p recognition sequence, leading to an accumulation of primary enveloped capsids in the perinuclear space. Our results suggest a new role of ORF9p in capsid nuclear egress, one of the crucial steps in herpesviruses infection.

FOOTNOTE
* corresponding author. Mailing address: Laboratory of Virology and Immunology, University of Liege, Avenue de l'Hopital 1, GIGA-R, B34 (+5), 4000 Liege, Belgium. Phone: (+32) 4 366 24 45. Fax: (+32) 4 366 41 98. E-mail: csadzot@ulg.ac.be
[$] actual address: Center for Infection & Immunity of Lille (CIIL), Lille Pasteur Institute, University Lille North of France, Lille, France.

60.

Sensitivity and permissivity of *Cyprinus carpio* to cyprinid herpesvirus 3 during the early stages of its development: importance of the epidermal mucus as an innate immune barrier

Ronsmans M.[1*], Boutier M.[1], Rakus K.[1], Farnir F.[2], Desmecht D.[3], Ectors F.[4], Vandecan M.[5], Lieffrig F.[6], Mélard C.[5] , Vanderplasschen A. [1]

[1]Immunology-Vaccinology, Department of Infectious and Parasitic Diseases (B43b), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium
[2]Biostatistics and Bioinformatics applied to Veterinary Science (B43), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium
[3]Pathology, Department of Morphology and Pathology (B43), Fundamental and Applied Research for Animals & Health, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium

[4]Transgenic Platform, Groupe Interdisciplinaire de Génoprotéomique Appliquée, Centre Hospitalier Universitaire B34, University of Liège, 4000 Liège, Belgium

[5]CEFRA-University of Liège, 10 Chemin de la Justice, 4500 Tihange, Belgium

[6]CERgroupe, 1 rue du Carmel, B-6900 Marloie, Belgium

* Correspondence:mronsmans@ulg.ac.be

Cyprinid herpesvirus 3 (CyHV-3) causes a lethal disease in common and koi carp (*Cyprinus carpio*). The present study investigated the ability of CyHV-3 to infect common carp during the early stages of its development (from embryos to fingerlings) after inoculation by immersion in water containing the virus. Fish were inoculated at different times after hatching with a pathogenic recombinant CyHV-3 strain expressing luciferase. The sensitivity and permissivity of carp to CyHV-3 were investigated using in vivo bioluminescence imaging. The susceptibility of carp to CyHV-3 disease was investigated by measuring the survival rate. Carp were sensitive and permissive to CyHV-3 infection and susceptible to CyHV-3 disease at all stages of development, but the sensitivity of the two early developmental stages (embryo and larval stages) was limited compared to later stages. The lower sensitivity observed for the early developmental stages was due to stronger inhibition of viral entry into the host by epidermal mucus. In addition, independent of the developmental stage at which inoculation was performed, the localization of light emission suggested that the skin is the portal of CyHV-3 entry. Taken together, the results of the present study demonstrate that carp are sensitive and permissive to CyHV-3 at all stages of development and confirm that the skin is the major portal of entry after inoculation by immersion in infectious water. The results also stress the role of epidermal mucus as an innate immune barrier against pathogens even and especially at the early stages of development.

61.

Human liver chimeric mice as a novel model for the study of hepatitis E virus infections

Ibrahim M. Sayed, Lieven Verhoye[1], Ali Farhoudi[1], Yannick Debing[2], Johan Neyts[2], Geert Leroux-Roels[1], Robert Purcell[3], Suzanne Emerson[3], Philip Meuleman[1]

[1]Center for Vaccinology, Ghent University, Ghent, Belgium
[2]Rega Institute, KUL, Leuven, Belgium
[3]Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA

The hepatitis E virus (HEV) is an enterically transmitted virus that belongs to the *Hepeviridae* family and is subdivided into 4 genotypes (gt 1-4). HEV of gt 1 and gt 2 are mainly restricted to developing countries and can only infect humans. Genotype 3 and 4 viruses can also infect pigs and represent an emerging problem in industrialized areas, in particular in Europe. HEV usually causes an acute self-limited infection but in people with suppressed immunity, caused by pregnancy, disease or immunosuppressive therapy, the infection can evolve to chronicity. Pregnant women, people with underlying liver disease and immune suppressed patients are at risk of developing severe and sometimes fatale complications. HEV infection can be treated with ribavirin (RBV) and/or interferon, but these molecules are contra-indicated in certain patient groups. In addition HEV isolates with decreased sensitivity to RBV have recently been identified. Therefore new antiviral molecules are needed for the treatment of HEV infection.

Although HEV can be cultured *in vitro* it can only be studied *in vivo* in non-human primates. However, the use of these animals is hampered by financial and ethical concerns. Therefore we wanted to evaluate whether mice of which the liver is largely repopulated with primary human hepatocytes are susceptible to HEV infection.

uPA$^{+/+}$-SCID mice transplanted with human hepatocytes were inoculated with different preparations of HEV of gt 1 or gt 3. A first group of mice was intrasplenically injected with a filtered stool suspension from a gt 1 HEV-infected chimpanzee (Sar Strain). A second group was inoculated intrasplenically with cell culture-derived HEV (HEVcc) of gt 3. A third group received an oral dose of HEVcc via gavage. Non-transplanted mice inoculated with the same preparations served as negative controls. Plasma and stool samples were collected weekly and viral RNA was quantified using an in-house qRT-PCR.

HEV RNA could be detected in plasma and stool of the mice from group 1. The viral titer increased over time reaching levels up to 1.54×10^7 IU/ml in plasma and 4.8×10^8 IU/ml in 10% (w/v) stool suspension. The viral load was significantly higher in the stool than in the plasma. In some mice the virus was only detectable in stool. Interestingly, naïve humanized mice became infected after intrasplenic injection of a stool suspension derived from HEV RNA positive mice, indicating the presence of infectious viral particles. Upon RBV therapy (50 to 100 mg/kg daily) a rapid drop in viral load was observed, resulting in HEV negativity within about 2 weeks. After cessation of therapy viral rebound was observed.

While HEVcc-inoculated mice (group 2) became actively infected, the viral load in stool was relatively low compared to the group 1 mice. The viral load in plasma remained below the limit of quantification. HEV RNA could not be detected in control mice and in mice that were orally exposed to HEVcc (group 3).

In conclusion, human liver chimeric mice can be infected with HEV. This small animal model will be a valuable tool for the *in vivo* study of HEV and the evaluation of novel antiviral molecules.

62.

A phosphoproteome analysis at the host-Respiratory syncytial virus interphase

Koen Sedeyn[1,2], Bert Schepens[1,2], Liesbeth Vande Ginste[1,2], Walter Fiers[1,2], Xavier Saelens[1,2]

1 Inflammation Research Center, VIB, Technologiepark 927, Ghent 9052, Belgium
2 Department of Molecular Protein Research, Ghent University, 9052 Ghent, Belgium

Human respiratory syncytial virus (HRSV) is an enveloped, negative single stranded RNA virus belonging to the *Paramyxoviridae* family and a common human respiratory pathogen. Although an HRSV infection is commonly associated with only mild respiratory symptoms, very young children and the elderly can be at risk for developing (severe) bronchiolitis or pneumonia. By the age of 2 years, almost all children have been infected with HRSV at least once, requiring hospitalization in 1-2% of the cases. Despite 60 years of research and the importance of this pathogen, no licensed HRSV vaccine or antiviral therapy are available. Only a humanized monoclonal antibody (Palivizumab, Synagis®) is used prophylactically in high risk infants. At present, very little is known about the cellular factors that counteract or are needed for HRSV infections. Just recently, cell surface expressed nucleolin was identified as the cellular HRSV receptor (Tayyari *et al*, 2011, nature medicine), illustrating how little is still known on the interplay between the host cell and HRSV. In this project we are investigating kinase-controlled signaling pathways of the host that either enhance or counteract HRSV infections. We believe that elucidating these pathways will help to understand the infection process in more detail and help to discover novel therapeutic targets. To investigate kinase controlled signaling pathways, we first performed a siRNA based knockdown screen of the complete human kinome (719 genes) in A549 cells. Replication of HRSV-A2 was determined by staining of HRSV plaques and validated afterwards by quantifying released HRSV virions in a kinetics setting. In a second approach that we are currently setting up, we want to determine and quantify changes in protein phosphorylation following HRSV infection by phospho-proteomics. TiO_2 enriched phosphorylated peptides from mock and HRSV infected A549 cells will be compared by label-free proteomics. Knockdown of 2 kinases resulted in reduced HRSV replication and this reduction was evident after knockdown with different siRNAs targeting these kinases. This suggests that these kinases play an important role during a HRSV infection and that the observed reductions in HRSV replication are the result of the specific knockdown of these 2 kinases and not due to off-target silencing. A MTS based metabolic activity assay excluded the possibility that the reduced HRSV replication after knockdown of these 2 kinases was due to a general decrease in metabolic activity. We also confirmed that a HRSV-Nucleoprotein siRNA (ALN-RSV01) clearly abolished HRSV replication. Currently we are trying to elucidate at which stage of the HRSV infection cycle these kinases might play a role.

63.

Evasion of cytotoxic T cell response by *Alcelaphine herpesvirus 1* genome maintenance protein.

Sorel O.[1], Myster F.[1], Vanderplasschen A.[1] et Dewals B.G.[1]

[1.] Immunology-Vaccinology, Department of infectious and parasitic diseases B43b, FARAH, Faculty of Veterinary Medicine, Boulevard de Colonster 20, University of Liège, B-4000, Liège - Belgium
Corresponding author : oceane.sorel@ulg.ac.be

Alcelaphine herpesvirus 1 (AlHV-1) is a gammaherpesvirus carried by wildebeest (*Connochaetes taurinus*) asymptomatically. After transmission to a variety of ruminant susceptible species, AlHV-1 is responsible for the development after a long incubation period of an acute and fatal disease, namely malignant catarrhal fever (MCF). MCF is characterized by the infiltration and proliferation of infected $CD8^+$ T cells in many organs. Recent studies have demonstrated that viral latency is essential during the disease. Among the rare transcripts detected during MCF, ORF73 is highly expressed. ORF73 encodes AlHV-LANA (for *latency-associated nuclear antigen*), an orthologous protein of the genome maintenance protein conserved in γ-herpesviruses. The high protein expression of AlHV-LANA should therefore induce an adaptive immune response. Even though such a response could exist, it is not protective since infected animals develop MCF. Genome maintenance proteins are responsible for the maintenance of viral episomes in latency and have *cis*-acting immune evasion properties. Here, we tested the capability of the AlHV-LANA to escape the cytotoxic T cell response by inhibiting its own presentation by the major histocompatibility complex class-I (MHC-I). We observed that AlHV-LANA was able to inhibit the MHC-I-restricted presentation to the cell surface of an OVA peptide fused to it, a mechanism involving canonic proteasomal pathways. Using targeted mutagenesis, we further demonstrated that the deletion of the GE-rich domain of AlHV-LANA restored the peptide presentation. The GE-rich domain was associated with reduced steady-state expression of the protein, an observation that could be correlated with reduced translation efficacy as well as reduced mRNA transcription levels. In conclusion, we demonstrated that the GE-rich domain of AlHV-LANA is involved in the *cis*-acting immune evasion properties, putatively through a mechanism inhibiting protein expression and mRNA transcription. Future work should determine whether this mechanism enables latently infected cells to evade the immune system of the infected host.

64.

The ORF27 gene of Cyprinid herpesvirus 3: A case of opposite selective pressures *in vitro* and *in vivo*?

Vancsok C.[1], Jazowiecka-Rakus J.[1], Boutier M.[1], Reschner A., Rakus K.[1], Vanderplasschen A.[1]

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

Cyprinid herpesvirus 3 (CyHV-3) is the aetiological agent of a lethal disease in common and koi carp. Sequencing of its genome revealed the **ORF25 family, a family of type I membrane proteins** composed of 6 paralogous sequences: ORF25, ORF26 (pseudogene), ORF27 (described as a pseudogene in laboratory strain), ORF65, ORF148 and ORF149. ORF25, ORF65, ORF148 and ORF149 encode structural protaines. The present study was devoted to the study of ORF27. First, to determine whether ORF27 encodes a structural protein, a FL BAC recombinant viral strain encoding a wild type ORF27 sequence was produced using the FL BAC laboratory strain as parental background. Mass spectrometry analyses of purified virions demonstrated that **ORF27 encodes a structural protein**, thereby extending the ORF25 family to 5 paralogous structural envelope proteins. Second, we investigated whether ORF27 mutation occurring *in vitro* led to the secretion of expression product or to its non expression. Sequence alignments were performed on several strains of CyHV-3, showing that mutations in ORF27 do not systematically occur in the transmembrane domain, suggesting that the selection of ORF27 mutation in vitro are associated with its non expression. Furthermore, in order to allow pORF27 detection and, to a broader extent, to allow further study of the ORF25 family paralogues, polyclonal sera were produced against each paralogue using DNA vaccination. Immunostaining of cells expressing the paralogues with the sera produced revealed no cross-reaction between the members of the family. Based on these results, immunofluorescent stainings were performed with the serum against pORF27 on cells infected with different strains of CyHV-3. Confocal microscopy analyses revealed a stronger pORF27 detection in strains encoding a wild-type ORF27 compared to strains encoding a truncated ORF27. Taken together, these results suggest that the **mutation occurring *in vitro* leads to the non expression of pORF27**, suggesting that **ORF27 influences negatively viral growth *in vitro***.

The next step of the project consists to investigate whether ORF27 could influence positively viral growth and/or transmission *in vivo* implying **opposite selective pressures *in vitro* and *in vivo***. Four recombinant viral strains will be produced. On one hand, strains encoding a wild-type ORF27 and on the other hand, strains encoding a truncated ORF27. For each genotype, two sets will be produced: one encoding a luciferase expression cassette, and one that does not express a reported gene. The use of luciferase positive variants will allow *in vivo* detection of the virus using *In Vivo* Imaging System. These four recombinant viral strains will be compared using two different strategies: infections with a single virus *versus* co-infection to address viral competitive fitness.

65.

HIV triggers a cGAS-dependent type 1 IFN response in primary CD4$^+$ T cells that is regulated by Vpu and Vpr

Jolien Vermeire[1], Anouk Van Nuffel[1], Ferdinand Roesch[2], Daniel Sauter[3], Hanne Vanderstraeten[1], Evelien Naessens[1], Veronica Iannucci[1+], Alessia Landi[1], Wojciech Witkowski[1], Ann Baeyens[1], Olivier Schwartz[2], Frank Kirchhoff[3], Bruno Verhasselt[1*]

[1]Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University, Ghent, Belgium
+ present address: Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA
[2]Institut Pasteur, Unité Virus et Immunité, Département de Virologie, Paris, France
[3]Institute of Molecular Virology, Ulm University Medical Center, 89081 Ulm, Germany

Background
Production of type 1 interferon (IFN) in response to viral infection requires detection of viral replication products by cellular pattern recognition receptors. HIV is capable of inducing an elaborate IFN response in plasmacytoid dendritic cells (pDCs). However in T cells and macrophages, the main HIV target cells, IFN induction by HIV is more controversial. Here, we re-evaluate the occurrence and underlying mechanism of an innate immune response upon HIV infection of primary CD4$^+$ T cells.

Methods and Results
Type 1 IFN induction was evaluated in activated purified CD4$^+$ T cells during productive HIV-1 infection. We observed an increase in IFN-β, IFN-α and different interferon stimulated genes (ISGs) by HIV-1 NL4.3 and different HIV-1 and HIV-2 primary isolates. Addition of IFN-β and IFN-α neutralizing antibodies to the HIV cultures decreased ISG induction and enhanced HIV-1 replication, indicating that HIV-induced IFN has an antiviral effect. To gain more insight in the mechanism of IFN induction by HIV-1, we used inhibitors of different HIV replication steps and found that integration of the HIV provirus is required for full IFN induction in CD4$^+$ T cells. In agreement with this, IFN induction was not observed when using an *integrase*-mutated or a *tat*-mutated HIV-1 virus. This indicates that production of new HIV RNA and/or proteins is required for evoking an innate immune response in infected CD4$^+$ T cells. Therefore, we subsequently evaluated the role of different HIV accessory proteins in IFN induction. Use of *vpr*- or *vpu*-deleted viruses showed a stimulating effect of Vpr and an inhibiting effect of Vpu on IFN response. Finally, knock-down of different host IFN pathway proteins with shRNA-encoding lentiviral vectors, indicated the requirement of the DNA sensor cGAS and its downstream signaling molecules for IFN induction.

Conclusion
Our data show that activated CD4$^+$ T cells are capable of producing relevant levels of type 1 IFN in response to HIV infection and suggest a post-integration sensing of HIV DNA by cGAS as a trigger of IFN induction in these cells. Based on this, we speculate that cGAS sensing of HIV RT products is enabled in activated CD4$^+$ T cells through the assistance of a newly expressed HIV-1 protein, possibly Vpr, and Vpu might have evolved to partially counteract this phenomenon.

66.

Age and strain dependent differences in the outcome of experimental infections of domestic pigs with Belgian wild boar pseudorabies virus isolates

Sara Verpoest[1], Ann Brigitte Cay[1], Herman Favoreel[2], Nick De Regge[1]

[1] CODA/CERVA, Groeselenberg 99, 1180 Ukkel, Belgium ; [2] Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Aujeszky's disease is an economically important disease in domestic swine caused by the porcine alphaherpesvirus, pseudorabies virus (PRV). Although the virus is eradicated in domestic pigs in a large part of Europe, serological studies show that the virus is still circulating in wild boars. Infection studies suggest an attenuated nature of these wild boar strains, but care should be taken when extrapolating these results since phylogenetic studies identified the existence of important genetic diversity among these strains.

To get more insight into the virulence of Belgian wild boar PRV strains, an infection study using two genetically distinct wild boar isolates and the NIA3 reference strain was performed. Six female domestic pigs of 15 weeks old were intranasally inoculated with 10^5 TCID50 of the NIA3 strain or 10^6 TCID50 of the wild boar isolates BEL24043 or BEL20075. Furthermore, six contact animals were added to the boxes at 24 hpi to assess virus transmission. Since clinical disease induced by PRV is age dependent, also six animals of two weeks old were inoculated with the NIA3 strain or the isolate BEL24043. At regular time intervals, temperature and clinical symptoms were determined and serum, nasal and vaginal swabs were collected. Animals were euthanized and tissues collected at 28 dpi.

A clear difference between clinical symptoms induced by the NIA3 strain and the wild boar isolates BEL24043 and BEL20075 was observed. In 15 weeks old pigs, infection with the NIA3 strain led to severe respiratory and neurological symptoms whereas no clinical symptoms were observed after inoculation with the isolates BEL24043 or BEL20075. In two weeks old piglets, the symptoms induced by the NIA3 strain were even more pronounced, but also the isolate BEL24043 induced respiratory and neurological disease, although less severe compared to NIA3 infection. Inoculation of 15 weeks old pigs with the NIA3 strain and both wild boar isolates as well as inoculation of two weeks old piglets with the isolate BEL24043 resulted in seroconversion. PCR analysis of swabs showed a prolonged nasal and vaginal virus excretion after inoculation of 15 week old pigs with the isolate BEL24043. Importantly, efficient transmission of the virus to contact animals was evidenced by nasal and vaginal excretion, seroconversion and the presence of virus in the tonsils of these contact animals. On the other hand, transmission of isolate BEL24043 was only evidenced to one contact animal.

In conclusion, our study identified differences between Belgian wild boar isolates in their capacity to infect and spread between domestic pigs. The difference in clinical symptoms in two and 15 weeks old piglets showed that the known age dependency of pigs towards the outcome of infection with PRV isolates from domestic pigs is also valid for wild boar strains. Although both wild boar strains were attenuated in 15 weeks old pigs, one was capable to induce seroconversion and was efficiently transmitted to contact animals. Despite the fact that reports of PRV transmission from wild boars to the domestic population are rare, this indicates that a reintroduction could have serious economic consequences.

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67.

HIV-1 integrase/Pol recruits LEDGF/p75 into viral particles

Weydert C.1, Desimmie B.1†, Schrijvers R.1, Vets S.1, Demeulemeester J.1,Proost P.2, Paron I.3, Mast J.4, Bannert N.5, Gijsbers R.1, Christ F.1, De Rijck J.1 and Debyser Z.1

1 KU Leuven, Division of Molecular Virology and Gene Therapy, Leuven, 3000, Belgium 2KU Leuven, Laboratory of Molecular Immunology, Rega Institute, Leuven, Flanders, Belgium 3Department of Proteomics and Signal Transduction, Max-Planck Institute of Biochemistry, D-82152 Martinsried, Germany. 4Veterinary and Agrochemical Research Centre CODA-CERVA, Brussels, Belgium 5Robert Koch Institute, Centre for HIV and Retrovirology, Berlin, Germany †Present address: Viral Mutation Section, HIV Drug Resistance Program, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

During Human Immunodeficiency Virus (HIV) infection the viral integrase stably integrates the cDNA into the host cell chromatin. HIV is tethered to the chromatin by the cellular protein LEDGF/p75. In 2010 a novel class of small molecules (LEDGINs) was described that potently block HIV integration through the inhibition of the interaction of LEDGF/p75 with integrase and through stimulation of integrase multimerization. However, recent studies revealed that LEDGINs also impair the infectivity of newly produced virions (late effect). Additionally, LEDGF/p75 binding peptides blocking the integrase interaction, showed a similar phenotype. These data, together with the discovery of LEDGF/p75 as a binding partner of the HIV Pol precursor protein, made us hypothesize that LEDGF/p75 is recruited by integrase/Pol into viral particles and hence, contributes to the potency of LEDGINs.
To address this hypothesis, viral particles were purified using an iodixanol velocity gradient and the presence of LEDGF/p75 in lysates from pure viral particles was analyzed by immunoblotting. LEDGF/p75 fragments, but not full length LEDGF/p75, were detected in lysates from ultrapure particles. Detection of full length LEDGF/p75 upon addition of ritonavir, a HIV protease inhibitor, during production and purification, suggested that LEDGF/p75 is a substrate for HIV protease. An *in vitro* protease assay confirmed this finding. Furthermore, no LEDGF/p75 fragments were detected in lysates from ultrapure viruses containing an integrase mutant defective for LEDGF/p75 binding which supports the specific incorporation of LEDGF/p75 by HIV integrase. Next, AlphaScreen and pull down assays confirmed interaction of LEDGF/p75 with the Pol precursor protein suggesting that incorporation can be mediated by Pol. To investigate whether intravirion LEDGF/p75 contributes to LEDGIN potency, virions were produced in the presence of a dilution series of compound in LEDGF/p75 knock down cells. The compounds were equally active against virus produced on LEDGF/p75 knock down and wild type cells, revealing no major contribution of intravirion LEDGF/p75 to late stage LEDGIN potency and assigning the late stage potency of LEDGINs to integrase multimerization. Furthermore, the infectivity of viruses produced on LEDGF/p75 knock out cell lines was only slightly hampered.
In conclusion, we demonstrate that LEDGF/p75 is incorporated specifically by HIV integrase/Pol into viral particles and that LEDGF/p75 is a substrate of HIV protease. The presence of intravirion LEDGF/p75 is not crucial for LEDGIN potency. Further experimentation is required to elucidate the role of intravirion LEDGF/p75.

68.

shRNA-mediated gene knock-down in monocyte derived dendritic cells identifies host factors affecting HIV infection

Wojciech Witkowski, Jolien Vermeire, Alessia Landi, Evelien Naessens, Hanne Vanderstraeten, Bruno Verhasselt

Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, De Pintelaan 185, Gent, Belgium

Understanding early events in the sexual HIV transmission is important for developing successful prevention and treatment strategies. Dendritic cells (DCs) play a crucial role in this process by transmitting viral particles from the mucosae to local lymph nodes where systemic infection is established. Accumulating body of evidence suggests, that apart from being purely a mean for viral transport and dissemination, DCs shape immune responses to HIV. Our study aims at identification of host factors expressed in DCs affecting HIV-1 infection and transmission to $CD4^+$ T cells. To this end, we have set-up an *in vitro* culture system that allows for selective knock-down of gene expression in monocyte derived dendritic cells (MDDCs) by lentiviral shRNA transduction. We have validated the model by successful knock-down of HIV entry receptor CD4 and resistance factor SAMHD1 which resulted in blocking and increasing infection respectively. Consequently we have investigated downregulation of several genes involved in regulation of actin cytoskeleton, endocytosis and pathogen sensing. We have identified three potential factors: ARHGAP24, ATP6V1B1 and FNBP1L, which upon knock-down led to marked increase of infection in MDDCs. We were able to confirm this findings in different blood donors, and could reproduce similar effects in a T cell line (Jurkat E6.1). Data obtained so far suggests, that genes of interest function at early stages of the viral life cycle, independently of the viral accessory protein Nef.

69.

A murine model for studying primary cytomegalovirus (CMV) infections: characteristics of the pathogenesis of murine CMV infections in neonatal and adult mice using a new Belgian isolate

Jun Xiang[†], Shunchuan Zhang[†], Hans Nauwynck ([†]contributed equally)

Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

In healthy individuals, naturally acquired infections of human cytomegalovirus (HCMV) are generally asymptomatic. The animal models that mimic the natural primary HCMV infection are scarce. Here, we developed a murine model for primary infections of HCMV. Neonatal (3 days old) and adult (8 weeks old) Balb/c mice were inoculated oronasally with 10^5 $TCID_{50}$ of a Belgian isolate HaNa1 of murine cytomegalovirus (MCMV). None of the mice showed clinical signs throughout the experiment. Initial viral replication occurred in the nose of both age groups by 3 days post inoculation (dpi). From 5 till 17 dpi, virus was replicating in the lungs of the neonates. In adult mice, virus was detected in the lungs of only one out of three animals at one time point-5dpi. From 7 dpi, a systemic infection was developed in neonatal mice. In adult mice, the infection stayed restricted to the nose and submandibular salivary gland. Infectious virus was never detected in trachea, oral mucosa, pharynx, esophagus, small intestines, and plasma of neonatal and adult mice. Adult and neonatal mice raised MCMV-specific immunoglobulin G (IgG) from 10 dpi and from 14 dpi, respectively. The IgG antibody titer and avidity increased with time. In summary, a murine model for natural primary HCMV infections was developed, which mimics the pathogenesis and clinical outcome of natural primary HCMV infections in healthy humans. This new model will be used in the future to shed further light on the pathogenesis and to develop vaccines against HCMV.

70.

A Beneficiary Role for Neuraminidase in Influenza Virus Penetration through the Respiratory Mucus

Xiaoyun Yang, Lennert Steukers, Katrien Forier, Ranhua Xiong, Kevin Braeckmans, Kristien Van Reeth, Hans Nauwynck

Abstract

Swine influenza virus (SIV) has a strong tropism for pig respiratory mucosa, which consists of a mucus layer, epithelium, basement membrane and lamina propria. Sialic acids present on the epithelial surface have long been considered to be determinants of influenza virus tropism. However, mucus which is also rich in sialic acids may serve as the first barrier of selection. It was investigated how influenza virus interacts with the mucus to infect epithelial cells. Two techniques were applied to track SIV H1N1 in porcine mucus. The microscopic diffusion of SIV particles in the mucus was analyzed by single particle tracking (SPT), and the macroscopic penetration of SIV through mucus was studied by a virus in-capsule-mucus penetration system, followed by visualizing the translocation of the virions with time by immunofluorescence staining. Furthermore, the effects of neuraminidase on SIV getting through or binding to the mucus were studied by using zanamivir, a neuraminidase inhibitor (NAI), and *Arthrobacter ureafaciens* neuraminidase. The distribution of the diffusion coefficient shows that 70% of SIV particles were entrapped, while the rest diffused freely in the mucus. Additionally, SIV penetrated the porcine mucus with time, reaching a depth of 65 µm at 30 min post virus addition, 2 fold of that at 2 min. Both the microscopic diffusion and macroscopic penetration were largely diminished by NAI, while were clearly increased by the effect of exogenous neuraminidase. Moreover, the exogenous neuraminidase sufficiently prevented the binding of SIV to mucus which was inversely enhanced by the effect of NAI. These findings clearly show that the neuraminidase helps SIV move through the mucus, which is important for the virus to reach and infect epithelial cells and eventually become shed into the lumen of the respiratory tract.

71.

The envelope glycoprotein gp150 promotes sexual transmission of Murid herpesvirus 4.

Caroline Zeippen [1], Alain Vanderplasschen [1], Philip Stevenson [2], Laurent Gillet [1]

[1]Laboratory of Immunology-Vaccinology, FARAH, University of Liège, Belgium.
[2]Sir Albert Sakzewski Virus Research Centre, Queensland Children's Medical Research Institute and Australian Infectious Disease Research Centre, University of Queensland and Royal Children's Hospital, Brisbane, Queensland 4029, Australia.

Gammaherpesviruses are important pathogens in human and veterinary medicine. During co-evolution with their hosts, they developed many strategies allowing them to shed infectious particles in presence of immune response. Understanding these strategies is likely to be important to control infection. Interestingly, we recently observed that Murid herpesvirus 4 (MuHV-4), a gammaherpesvirus infecting laboratory mice, could be sexually transmitted between mice. This model offers therefore the opportunity to test the importance of immune evasion mechanisms during transmission. One of these mechanisms could rely on the glycoprotein 150 (gp150) which seems to be used as an immunogenic decoy that could limit virus neutralization. Indeed, while gp150 is the main target of antibodies raised against MuHV-4, anti-gp150 antibodies are not neutralizing but instead enhance Fc-receptor dependent infection *in vitro*. Furthermore, gp150 could form a glycan shield at the surface of the virion that limits neutralization and could promote the release of infectious particles from infected cells. In this study, we tested therefore the importance of gp150 in the context of MuHV-4 sexual transmission. Briefly, female mice were infected with strains expressing luciferase and deficient or not for the expression of gp150. At different times post-infection, these mice were imaged with an *in vivo* imaging system to follow the lytic infection. Finally, at the moment of lytic replication in the genital tract, infected females were mated with naïve males to compare the capacity of transmission of the two strains. Our results show that, while the gp150- strain has no deficit in infection or in genital excretion compared to the wild-type strain, the gp150- strain displays a major deficit of sexual transmission. Interestingly, this deficit does not result from an increased sensitivity to antibody neutralisation but seems to reflect a release deficit of virions from vaginal epithelial cells. Altogether, our results show that, while gp150 is not required for efficient dissemination and maintenance of MuHV-4 within its host, it is essential for efficient transmission, at least by the sexual route.

72.

Comparison of the pathogenesis of the highly passaged MCMV Smith strain with that of the low passaged MCMV HaNa1 strain in Balb/c mice upon oronasal inoculation

Shunchuan Zhang[†], Jun Xiang[†], Hans Nauwynck ([†]contributed equally)

Laboratory of Virology, Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Abstract

Murine cytomegalovirus (MCMV) Smith is widely used to set up animal models to study CMV infections. However, due to the high number of *in vitro* passages, MCMV Smith has acquired unwanted genetic and biological changes. Therefore, a low passaged strain would be much better to develop animal models. Here, the pathogenesis of mice inoculated with MCMV Smith was compared with that of a low passaged Belgian MCMV isolate (HaNa1) in BALB/c adult mice following oronasal inoculation with either a low (10^4 $TCID_{50}$ per mouse) or a high (10^6 $TCID_{50}$ per mouse) inoculum. At regular time points post inoculation (pi) mice were euthanized and tissues were collected for virus titration, co-cultivation and immunofluorescence staining. Plasma was analyzed by an immunoperoxidase monolayer assay (IPMA) for the detection of virus-specific antibodies as well as antibody classes and subclasses, and by a complement-dependent neutralization test for the detection of neutralizing antibodies.

Both strains (HaNa1 and Smith) were mainly replicating in the nose and submandibular salivary glands. In the nose, MCMV was detected earlier and longer, and reached higher titers with the high dose inoculum (from 1dpi till the end of the experiment 49dpi) compared to the low dose (from 14dpi till 35dpi). In submandibular glands, a similar observation was done (low dose: from 14 till 42dpi/high dose: from 7 till 49dpi). In lungs, MCMV showed a very restricted replication. Both strains induced a cell-associated viremia at 7-10dpi and cell-associated virus was present in the nasal-associated lymphoid tissue (1-49dpi, end of the experiment), submandibular lymph nodes (3-5dpi), cervical lymph nodes (3-21dpi) and spleen (5-21dpi). Only with the Smith strain, infectious virus was isolated from internal organs (spleen, liver and kidneys) during the second and third week of infection. Viral antigen positive cells were detected for both strains in the nasal-associated lymphoid tissue, olfactory nasal mucosa, submandibular salivary glands and lungs. Antibody analysis demonstrated that increasing the inoculation dose shortened the time of appearance of viral-specific (IPMA) and neutralizing antibodies, and also increased their level for both strains. IgG_{2a} was the main antibody subclass that was detected for both strains and doses. Overall, our results show that HaNa1 is causing a local replication in nose/salivary glands whereas the Smith strain is causing in addition a generalized productive infection. Therefore, the HaNa1 strain is mimicking better the replication of HCMV in immunocompetent hosts and is thus more suitable for use in animal models.