Third annual meeting
Belgian Society for Virology
Friday, December 18, 2015
The Royal Academies for Science and the Arts
Hertogsstraat/Rue Ducale 1, Brussels

8h45: Welcome & Belgian Society for Virology - General Assembly

9h00-9h45: Keynote lecture 1 - Andrew Davison "Herpesviral pathogenomics"

9h45-12h30 Presentations-Pathogenesis/immune response/immune evasion-viral diseases

9h45-10h00: Selected talk 1
Role of Sp1 and CTIP2 in the transcriptional regulation of Human T-Lymphotropic Virus-1 (HTLV-1)
Fauquenoy Sylvain, Gwenaëlle Robette, Anna Kula, Nadège Delacourt, Caroline Vanhulle, Anthony Rodari, Arsène Burny, Benoît Van Driessche and Carine Van Lint.

10h00-10h15: Selected talk 2
MCMV exploits the spleen as a transfer hub for systemic dissemination upon oronasal inoculation
Shunchuan Zhang, Jun Xiang, Sebastiaan Theuns, Lowiese M.B. Desmarets, Hans J. Nauwynck

10h15-10h45: Coffee break

10h45-11h00: Selected talk 3
Role of hepatitis C virus in hepatocellular carcinoma-associated angiogenesis
Mohammed Benkheil, Jan Paeshuyse, Mareike Grabner, Johan Neyts, Sandra Liekens

11h00-11h15: Selected talk 4
Antibody-induced internalizaton of RSV F proteins 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

11h15-11h30: Selected talk 5
Theiler's virus L* protein inhibits RNase L dimerization and activation by binding to the 2-5A-binding pocket of the enzyme
Melissa Drappier , Babal K. Jha, Robert H. Silverman, Thomas Michiels

11h30-12h30: Speed presentations (5 minutes each/without discussion)

1. Visualisation of the human immunodeficiency virus type 1 cDNA by click chemistry
Flore De Wit, Lieve Dirix and Zeger Debyser

2. *In vivo* characterization of the RNAPIII-dependent BLV miRNAs cluster
Anthony Rodari, Benoît Van Driessche, Nadège Delacourt, Caroline Vanhulle, Sylvain Fauquenoy, Mathilde Galais, Arsène Burny, Anne Van den Broeke, Olivier Rohr, and Carine Van Lint

3. Role of bovine leukemia virus micro-RNAs in replication and oncogenesis
Nicolas A. Gillet; Malik Hamaidia; Alix de Brogniez; Gerónimo Gutiérrez; Nathalie Renotte; Michal Reichert; Karina Trono and Luc Willems

4. Reassortment capacities of Simbuvirus closed to Schmallenberg virus
Damien Coupeau, Pierre Baillieux, Axel Marichal, Laetitia Wiggers, Nathalie Kirschvink and Benoît Muylkens.

5. Bovine herpesvirus 4 modulates its beta-1,6-N-acetylglucosaminyltransferase activity through alternative splicing
Lété C., Markine-Goriaynoff N., Machiels B., Pang P., Xiao X., Canis K., Suzuki M., Fukuda M., Dell A., Haslam S., Vanderplasschen A. and Gillet L.

6. Self-inhibition of synthesis reduces antigen presentation of the alcelaphine herpesvirus 1-encoded latency-associated protein, aLANA
Océane Sorel, Françoise Myster, Alain Vanderplasschen, Benjamin G. Dewals

7. Influenza A virus escape routes from immune selection by monoclonal antibodies directed against the ectodomain of matrix protein 2
Silvie Van den Hoecke, Lei Deng, Walter Fiers, Bert Schepens, Xavier Saelens

8. A herpesvirus alters the behavior of its host to enhance its replication
Maygane Ronsmans, Krzysztof Rakus, Maria Forlenza, M. Carla Piazzon, Geert F. Wiegertjes, Maxime Boutier, Joanna Jazowiecka-Rakus, Alekos Athanasiadis, Frédéric Farnir, Thomas Michiels and Alain Vanderplasschen

9. Relationship between cytokine expression and viral replication and spread during the early stages of pseudorabies virus infection in pigs.
Sara Verpoest, Ann Brigitte Cay, Herman Favoreel, Nick De Regge

10. Experimental feline enteric coronavirus infection reveals an aberrant infection pattern and shedding of mutational variants with impaired enterocyte tropism
Lowiese M.B. Desmarets, Ben L. Vermeulen, Sebastiaan Theuns, Nádia Conceição-Neto, Mark Zeller, Inge D.M. Roukaerts, Delphine D. Acar, Dominique A.J. Olyslaegers, Marc Van Ranst, Jelle Matthijnssens and Hans J. Nauwynck

11. Epigenetic regulation of gga-microRNA-126 during lymphoproliferative disease in chicken
Isabelle Gennart, Lore Parissi, Srdan Pejakovic, Benoît Muylkens

12. Gp150 promotes sexual transmission of Murid Herpesvirus-4.
Zeippen C., Javaux J., Xiao X., Farnir F., Vanderplasschen A., Stevenson P.G. and Gillet L.

12h30-13h30: Sandwich lunch

13h30-14h15: Keynote lecture 2 - Bart Haagmans "MERS coronavirus"

14h15-17h00: Presentations-Control of viral diseases (antivirals/vaccination)

14h15-14h30: Selected talk 1
New antivirals against hRSV
Iebe Rossey, Bert Schepens, Jason McLellan, Barney Graham, Xavier Saelens

14h30-14h45: Selected talk 2
The genome of a tortoise herpesvirus (testudinid herpesvirus 3) has a novel structure and contains a large region that is not required for replication *in vitro* or virulence *in vivo*
Frédéric Gandar, Gavin S. Wilkie, Derek Gatherer, Karen Kerr, Didier Marlier, Marianne Diez, Rachel E. Marschang, Jan Mast, Benjamin G. Dewals, Andrew J. Davison, Alain F.C. Vanderplasschen

14h45-15h15: Coffee break

15h15-15h30: Selected talk 3
LEDGIN mediated inhibition of the IN-LEDGF/p75 interaction reduces reactivation from latency
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A novel class of chikungunya virus inhibitors targets the viral capping enzyme nsP1
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16h00-17h00: Speed presentations (5 minutes each/without discussion)

1. Antibody dependent macrophage mediated phagocytosis and trogocytosis of influenza and HRSV infected cells, visualized by live cell imaging.
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2. A gammaherpesvirus infection protects from allergic asthma development
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3. Murid herpesvirus 4 infection protects mice from the development of pneumovirus induced immunopathologies
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4. Sequential Treatment with 5-aza-2'deoxycitidine and Deacetylase Inhibitors Reactivates HIV-1

Sophie Bouchat, Nadège Delacourt, Anna Kula, Gilles Darcis, Benoît Van Driessche, Francis Corazza, Jean-Stéphane Gatot, Adeline Melard, Caroline Vanhulle, Kevin Maerens, Kabamba Kabeya, Marion Pardons, Véronique Avettand-Fenoel, Nathan Clumeck, Stéphane De Wit, Olivier Rohr, Christine Rouzioux, and Carine Van Lint

5. Helminth-induced inflammation enhances cytotoxic T cell–mediated control of γ-herpesvirus infection.
Dougall A. M., Rolot, M., Vanderplasschen, A., Dewals, B.

6. Caps-It: An automated platform for multi-parameter data collection on live pathogens of higher or unknown biosafety risk.
Johan Neyts, Pieter Leyssen

7. An In-Depth Comparison of Latency-Reversing Agent Combinations in Various *in vitro* and *ex vivo* HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression.
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9. Enhanced pig-to-pig transmission of a reassortant H9N2 influenza virus containing 2009 pandemic H1N1 internal genes by serial passaging in pigs
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10. Oronasal immunization with low-passage MCMV HaNa1 in neonatal mice induces a strong protection against an infection with MCMV Smith in adulthood
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11. Checkpoint modulation by the human T-lymphotropic virus type 1 (HTLV-1) Tax protein: towards new therapeutic approaches
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1.

Involvement of the minichromosome maintenance 2-7 helicase in HTLV-1 transcription

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The human T-lymphotropic virus type 1 (HTLV-1) Tax protein interacts with the minichromosome maintenance MCM2-7 helicase. This interaction initiates early firing of late DNA replication origins and accelerates cell cycle progression. Since Tax binds the long terminal repeat (LTR), we hypothesized that the MCM2-7 complex could also be recruited to the viral promoter. Chromatin immunoprecipitations show that MCM2-7 binds to LTR sequences of integrated HTLV proviruses. Loading of the MCM2-7 complex does not support the replication of a LTR-containing plasmid in an autonomous replication assay. In contrast, MCM2-7 activates viral transcription in luciferase reporter assays. Mcm2-7 also increases viral transcription in the context of a complete HLTV provirus. Interference of MCM2-7 using shRNA inhibits LTR-driven Tax transactivation in lymphocytes. Finally, siRNAs targeting MCM3 affect viral transcription in HTLV-1 infected cell lines.
Together, our results demonstrate that the MCM2-7 complex is involved in HTLV transcription.

2.

Role of hepatitis C virus in hepatocellular carcinoma-associated angiogenesis

Mohammed Benkheil, Jan Paeshuyse, Mareike Grabner, Johan Neyts, Sandra Liekens

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Hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) shows a higher grade of vascularization compared to HCC with other underlying etiologies, suggesting that HCV may play a role in the establishment of a more angiogenic tumor microenvironment. However, little is known about how HCV contributes to HCC and HCC-associated angiogenesis. Angiogenesis or the formation of new blood vessels from pre-existing vessels is a major determinant of tumor malignancy since the newly formed vessels allow tumor progression and metastatic outgrowth. Thus, it is important to gain more insight into the potential role of HCV in this process.
Angiogenesis RT2 Profiler Array, quantitative RT-PCR and ELISA were performed to investigate the expression of angiogenic factors in HCV-infected human hepatoma Huh7 5.1 cells and in subgenomic replicon (SGR) cell lines encoding the non-structural (NS) proteins. Further, expression of angiogenic factors was validated in the absence and presence of the antiviral drugs Telaprevir (inhibitor of NS3/4A protease) and Sofosbuvir (inhibitor of the polymerase NS5B).
We found that the expression of several angiogenesis-regulating genes was significantly altered in HCV-infected Huh-7 cells and in hepatoma cells expressing HCV-SGR of both genotypes 1b and 2a. Furthermore, upregulation of angiogenic factors was normalized upon treatment with antiviral drugs, suggesting a direct role of HCV proteins. Moreover, observations from SGR cell lines indicate the involvement of HCV non-structural genes. Experiments using lentiviruses expressing individual HCV proteins are ongoing to identify the viral gene(s) responsible for the changes in the expression of angiogenic proteins.
Our data indicate that HCV may deregulate the angiogenic balance in HCC by increasing the expression of pro-angiogenic proteins. Stimulation of angiogenesis may be an additional mechanism by which HCV contributes to HCC development and/or malignancy.

3.

Sequential Treatment with 5-aza-2'deoxycitidine and Deacetylase Inhibitors Reactivates HIV-1

Sophie Bouchat[1], Nadège Delacourt[1], Anna Kula[1], Gilles Darcis[1,2], Benoît Van Driessche[1], Francis Corazza[3], Jean-Stéphane Gatot[1,$], Adeline Melard[4], Caroline Vanhulle[1], Kevin Maerens[1], Kabamba Kabeya[5], Marion Pardons[1], Véronique Avettand-Fenoel[4], Nathan Clumeck[5], Stéphane De Wit[5], Olivier Rohr[6,7], Christine Rouzioux[4], and Carine Van Lint[1,#].

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More than thirty years after its discovery, HIV-1 remains a major problem of public health. Persistence of truly latent (i.e. non-defective) HIV-1 proviruses represents a major obstacle to eradication. Indeed, the levels of HIV-1 reservoirs appear as one of the critical factors influencing the duration of a remission after cART cessation. Consequently, a decline of the HIV-1 latent reservoirs to a level sufficient to permit an efficient control of the infection by the host immune system might allow interruptions in therapy ("treatment-free windows"). Reactivation of HIV gene expression in latently-infected cells together with an efficient or intensified cART could serve as an adjuvant therapy aimed at eliminating/decreasing the pool of latent viral reservoirs. In this regard, results from HIV clinical trials using deacetylase inhibitors (HDACIs) question the efficiency of these latency reversing agents (LRAs) used alone and underline the need to evaluate other LRAs in combination with HDACIs.

In this report, we thoroughly studied the sequential administration of a DNA demethylating agent and clinically tolerable HDACIs first *in vitro* in latently-infected T-cell lines and next *ex vivo* in a large number (58) of cART-treated aviremic HIV-1^+ patient cell cultures. Interestingly, we showed that a sequential treatment with 5-AzadC and HDACIs was more effective than the corresponding simultaneous treatment to observe a synergistic activation of HIV production *in vitro* and *ex vivo*. Moreover, only two of the sequential combinatory treatments tested induced HIV-1 particle recovery in a higher manner than the LRAs alone in *ex vivo* patient cell cultures of $CD8^+$-depleted PBMCs. In conclusion, we reported for the first time that, in addition to the combinatory aspect, the time schedule of LRA treatment is critical to design purging strategies aimed at decreasing the HIV-1 reservoir size. Our results based on a molecular virology view clearly illustrate a clinically-focused translational research. We think that our study could interest and inspire HIV clinicians in order to elaborate clinical trial protocols. Indeed, this study clearly showed that the two promising combinations (5-AzadC + Panobinostat and 5-AzadC + Romidepsin) presented a reactivation potential at concentrations lower than the human tolerable plasmatic concentrations without presenting decreased cellular metabolic activity and global T-cell activation. Consequently, our findings should be beneficial for the design of future anti-latency therapeutic strategies and therefore constitute a step forward for HIV remission.

4.

The ORF57 protein of Cyprinid herpesvirus 3 is a major virulence factor

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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 vaccine. Recently, we showed that the double deletion of CyHV-3 ORF56 and ORF57 is associated with a safety/efficacy profile compatible with the use of this double deleted recombinant strain as an attenuated vaccine against CyHV-3 disease [1]. In the present study, we investigated the respective contribution of the two deleted genes to the attenuated phenotype observed. To address this question, a series of single deleted recombinants were produced. ORF56 and ORF57 were invalidated by replacement of sequences located in between predicted promoters of flanking genes by a *galK* cassette. The molecular structure of the recombinants produced was controlled. Immunofluorescence staining of carp cells infected with the different recombinants demonstrated that the deletion of ORF56 did not abrogate the expression of ORF57, and *vice versa*. These recombinants were further tested *in vivo* and revealed that the attenuation observed with the double deleted ORF56-57 resulted mainly from the deletion of ORF57.

This study demonstrates the role ORF57 as a major virulence factor of CyHV-3. As ORF57 is conserved amongst cyprinid herpesviruses, it could therefore represent a target for production of attenuated recombinant vaccine against these viruses that represent important fish pathogens.

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5.

Checkpoint modulation by the human T-lymphotropic virus type 1 (HTLV-1) Tax protein: towards new therapeutic approaches

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HTLV-1 is a retrovirus that infects at least 5 to 10 million people worldwide. HTLV-1 is the causative agent of adult T-cell leukemia (ATL) and a chronic neuropathology called HTLV-1 associated myelopathy / tropical spastic paraparesis (HAM/TSP). There is currently no satisfactory treatment for these diseases. We are interested in the mechanisms of cell transformation by the viral oncoprotein Tax. In particular, we aim at understanding the interplay between Tax and the DNA damage response (DDR). We show that transient expression of Tax results in DNA damage, cell cycle arrest and activation of the DDR. In fibroblasts, cell cycle arrest occurs at the G1 and G2 phases depending on the p53 background. In contrast, HTLV-1 infected lymphocytes proliferate continuously and appear to be adapted to checkpoint control. This mechanism of checkpoint adaptation thus allows ongoing proliferation despite the presence of genomic lesions. Furthermore, quantification of the rates of NHEJ, homologous recombination (HR) and single-strand annealing (SSA) indicates that HTLV-1 infected cells require very efficient and specific DNA repair pathways for survival. Therefore, we designed a novel therapeutic approach based on the use of inhibitors targeting DNA repair pathways.

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6.

Laboratory diagnosis of Ebola Virus Infection in Belgium

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Objective: During the past EVD epidemic in West-Africa, Belgium has been listed in the top four of European countries considered to be most at risk for importation of Ebola virus disease (EVD) outside Africa. In response to the latest EVD outbreak, the national reference laboratory for infectious and tropical diseases implemented EVD molecular tests to provide rapid diagnosis in Belgium.

Methodology: The Institute of Tropical Medicine (ITM) received permission on October 27th, 2014 by Federal and Flemish authorities to perform EVD diagnostics. Sample handling, malaria RDT testing, sample inactivation and nucleic acid extraction are performed in a dedicated BSL3+ laboratory, followed by real-time PCR testing for Plasmodium and for Ebola virus by targeting two different genes with an in-house and a commercial assay.

Results: Between October 2014 and September 2015, 17 samples from 8 suspected patients were analyzed. Six patients returned from Guinea, one from Sierra Leone and one from Liberia. All cases were EVD negative and one case tested positive for Plasmodium. One case of post-exposure vaccination with rVSV-ZEBOV GP after a needle-stick injury tested positive with the Ebola GP-specific and VSV-specific PCR. With the possibility of in-country testing, diagnostic delays were reduced significantly as compared to when sent to foreign laboratories. This enabled clinicians to lift strict isolation measures on average 1 day faster.

Conclusion: EVD testing was successfully implemented in Belgium and in-country testing enabled clinicians to lift isolation measures earlier. The two target-gene approach appeared to be necessary to avoid misinterpretation of results in patients vaccinated post-exposure.

7.

Reassortment capacities of Simbuvirus closed to Schmallenberg virus

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Abstract:
In 2011, a new Orthobunyavirus, the Schmallenberg virus (SBV) has been identified in Germany. Since then, SBV has been detected in several domestic and wild ruminants all over Europe. Phylogenetic analysis showed that SBV belongs to the genus *Orthobunyavirus,* within the family *Bunyaviridae* and is a member of the Simbu serogroup viruses. The segmented genome of SBV is composed of three negative single stranded RNA molecules, designated large (L), medium (M) and small (S) that altogether encode 6 proteins. Intramolecular recombination and reassortment are two ways to disseminate mutations in a viral population. *Orthobunyavirus* reassortants have been described by phylogenetic analyses but the genetic origin of SBV still unclear. Two viruses have been described to be related to SBV: (i) Shamonda virus (SHAV) containing S and L segments closely similar to the SBV respective segments; (ii) Sathuperi virus (SATV) whose M segment is close to the SBV M RNA.
In this study, we tested the *in vitro* recombination and reassortment capacities between the two Simbuvirus related to SBV (SHAV and SATV). SHAV and SATV were used to co-infect mammalian cells (BHK-21 cells) in two conditions of co-infection at low and high multiplicities of infection (M.O.I). A method was set up to generate, isolate and amplify clonal progeny viruses. Discriminative PCR were developed at both extremities (5' and 3') to determine the genetic background of the three segments in each progeny virions. Genomic characterization was completed with phenotypic assessment by using plaque size analyses, growth kinetics and stability of progeny virions over time. Moreover, competition assays were performed during ten successive infection passages in BHK-21 cells and evolution of the population has been determined after 1, 2, 5 and 10 passages.
No intramolecular recombination event has been identified but reassortment occurred at a high rate. Indeed, 26 % and 46.3 % of the viral progeny were reassortants viruses following the co-infections at low and high M.O.I respectively. In addition, the six potential reassortant configurations were generated in both conditions. Comparison of the growth kinetics and the stability of the isolated progeny viruses demonstrated their viability and their stability when propagated alone. Plaques size analysis showed a statistical difference among the different types of reassortants with largest plaques produced by viruses harboring the SHAV M segment. These viruses corresponded to the reassortants that were rapidly selected during competition assays.

8.

Mutation of a Single Envelope N-linked Glycosylation Site Enhances the Pathogenicity of Bovine Leukemia Virus

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Viruses have co-evolved with their host to ensure efficient replication and transmission without inducing excessive pathogenicity that would indirectly impair their persistence. This is exemplified by the bovine leukemia virus (BLV) system in which lymphoproliferative disorders develop in ruminants after 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 but fortunately, such a hyperpathogenic BLV strain was never observed in the field nor designed *in vitro*. In this study, we aimed at understanding the role of envelope N-linked glycosylation with the hypothesis that this posttranslational modification could either favor BLV infection by allowing viral entry or allow immune escape by using glycans as a shield. Using reverse genetics of an infectious molecular provirus, we have identified a N-linked envelope glycosylation site (N230) that limits viral replication and pathogenicity. Indeed, mutation N230E unexpectedly leads to enhanced fusogenicity and protein stability. Occurrence of this 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.

9.

Visualisation of the human immunodeficiency virus type 1 cDNA by click chemistry

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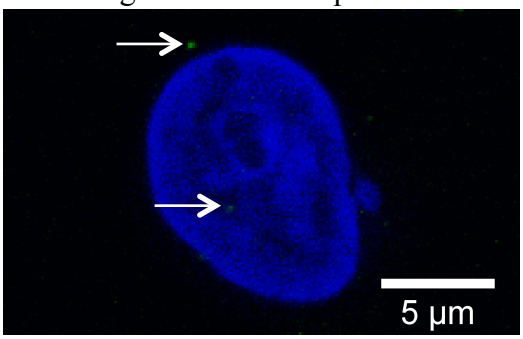
One of the key steps in the human immunodeficiency virus type 1 (HIV-1) replication cycle is the reverse transcription of viral RNA into double-stranded DNA carried out by the reverse transcriptase enzyme. This viral DNA is next transported, in the form of a pre-integration complex, into the nucleus where it can finally be integrated into the host chromosome by the viral integrase (IN). Although the process of reverse transcription is well known, many questions remain about the timing, location and the relation with nuclear import.
A method to study the HIV nuclear import was already developed in HeLa P4 cells with VSV-g pseudotyped IN-eGFP labeled HIV particles (Albanese *et al.*, 2008). We improved this method by using an HIV envelope to infect more relevant cell types, such as a T cell line, primary CD4+ T cells and monocyte derived macrophages. Although we can now identify single viral PICs in infected cells, we are unable to identify whether they contain viral DNA. Therefore, we combined our nuclear import assay with the labeling of viral DNA using click-chemistry. After addition of ethynyl-functionalised nucleosides during infection, they will be incorporated in the viral DNA by the reverse transcriptase. The ethynyl-functional group on the nucleosides allows covalent linkage with azide reactive fluorophores via a copper-catalyzed azide-alkyne cycloaddition. Since biological samples rarely contain alkynes or other functional groups reactive with azide, the labeling is highly specific. In 2014, Peng and colleagues showed that it is possible to image HIV cDNA with 5-ethynyl-2'-deoxyuridine (EdU) (Peng *et al.*, 2014*)*. However, we found that there are a few drawbacks to this technique. First of all there is a high unintentional labeling of the cytoplasm due to the incorporation of the nucleotide analogues by the host mitochondrial DNA polymerase and secondly, it is impossible to discriminate viral DNA in the nucleus due to the incorporation of the functionalized nucleotides by the host DNA polymerase. Therefore, we opt to develop reverse transcriptase specific ethynyl-functionalised nucleotides that are not incorporated by the cellular DNA polymerases. Eventually, we aim to establish an integrated imaging platform, by which the simultaneous detection of viral cDNA, IN-eGFP and capsid during the course of infection will allow a precise kinetic analysis of reverse transcription, trafficking, uncoating and nuclear import.



Nuclear import assay in primary CD4+ T cells.
CD4+ T cells were infected with HIV-IN-eGFP (green). PICs are highlighted by white arrows. DAPI staining was used to visualise the nucleus (blue).

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10.

HONEYBEE VIROMES REVEAL PRESENCE OF PATHOGENS AND NOVEL VIRUSES

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Introduction

Honeybees (*Apis Mellifera*) are domesticated social insects that play a crucial role in crop pollination. Management of honeybee colonies is performed to ensure crop pollination together with honey and propolis production. Honeybee colonies are in a worldwide decline and a large portion of this decline is due to a failure of a colony to survive the winter, a phenomenon called winter loss. This global decline coincides with the increasing agricultural demands for pollination of crops. In Belgium up to 30% of hives can be lost over the course of a single winter. Environmental factors, such as pesticide use, as well as various viruses alone or in combination with an infection with the *Varroa destructor* parasite have been suggested as risk factors for winter loss. Up to date, only a co-infection of deformed wing virus together with an infestation of the *Varroa destructor* parasite has been associated with winter loss. Considering the importance of honeybees in pollination, identification of pathogens associated with winter loss can help to elucidate the mechanisms behind winter loss and may allow the development of preventive strategies.

Methods

We used a metagenomics approach developed in our laboratory, specifically designed to sequence the genomes of enriched viral particles. We conducted a small pilot study using this method in an attempt to characterize the virome in healthy and diseased honeybees from three Flemish hives. Very briefly, viral particles were purified and extracted from entire honeybees. After reverse transcription and amplification, these were prepared for sequencing using the Illumina Hiseq2500 platform.

Results

Surprisingly, in addition to known pathogens such as Lake Sinai Virus, Filamentous virus and Varroa destructor virus-1, we identified a large number of highly divergent novel viruses. These new viruses are only very distantly related to Kashmir bee virus (35% amino acid similarity), Khasan virus (25% aa similarity), Bloomfield virus (31% aa similarity) and Kelp fly virus (26% aa similarity) among others. The complete genome of a distant relative of the Kashmir bee virus and the partial genome distantly related to Kelp fly virus are of particular interest since they belong to the order of the *picornavirales*. The vast majority of viruses known to be pathogenic for honeybees are members of this order. We are currently up scaling our metagenomics study to a higher sample size using Belgian honeybee samples collected during a large European study named Epilobee, in order to elucidate the role of such novel viruses in winter loss. This approach will allow us to fully characterize the virome in Belgian honeybees as well as identify viral pathogens involved in diseased honeybee colonies.

11.

A novel class of chikungunya virus inhibitors targets the viral capping enzyme nsP1

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Alphaviruses cause severe diseases such as encephalitis or arthritis in humans and animals. Some alphaviruses, such as the chikungunya virus (CHIKV), are in particular a significant global health threat, because of their high disease burden, their (re-)emergence, and the lack of vaccines or antiviral therapy. CHIKV is responsible for (ongoing) outbreaks in the Caribbean and the Americas and has caused large-scale outbreaks before in Africa and Asia. CHIKV infections are rarely fatal, but they progress in up to 60% of the patients into a chronic disease that is characterized by persistent disabling polyarthritis, which can severely incapacitate the patient for weeks up to years after the acute infection.
We recently identified a class of [1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones as selective inhibitors of CHIKV replication in cell culture. Using a representative analogue of this series, MADTP-314 [3-(3′-acetylphenyl)-5-methyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-one] we demonstrated activity against a variety of CHIKV strains, including a recent clinical isolate from the Caribbean. To elucidate the mechanism of the anti-CHIKV activity of this family of compounds, MADTP-314 drug-resistant CHIKV variants were selected. In all independently-selected drug-resistant CHIKV 899 variants, a single mutation was identified in the nsP1 coding sequence that resulted in a P34S amino acid substitution. Reverse-engineering confirmed the link between this mutation and the compound-resistant phenotype. Interestingly, the viral nsP1 protein is the main enzyme involved in viral mRNA capping and P34 is located in the N-terminal part of the protein, responsible for the methylation of GTP into m^7GTP (MTase), followed by the formation of a m^7GMP-nsP1 product (nsP1 guanylylation) and a subsequent transfer of m^7GMP to the RNA 5' end. Using biochemical assays with purified nsP1 of Venezuelan equine encephalitis virus (VEEV) as an *in vitro* capping model, we demonstrated that MADTP-314 inhibits the guanylylation of nsP1. Introduction of the corresponding MADTP-resistance mutation in VEEV nsP1 (D34S) abrogated the inhibitory effect of the MADTP compounds on the nsP1 capping functions.
We demonstrate here, to the best of our knowledge, for the first time that the capping machinery of alphaviruses is an excellent target for inhibition of alphavirus replication. Considering the lack of therapeutic options to treat infections with this re-emerging virus, the MADTP series of compounds with their unique (alphavirus-specific) target provide a promising starting point to develop therapy and/or prophylaxis for CHIKV infections.

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12.

Current status of the association of BoHV-4 with bovine abortions in southern part of Belgium

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ABSTRACT
Abortions cause heavy economic losses for the bovine sector. The use of a standardized panel of analyses covering a large spectrum of pathogens responsible of abortion in cattle, allowed demonstrating the direct involvement of at least one pathogen in 57% of analysed abortions in southern part of Belgium. This result suggests a margin of improvement in the diagnostic efficacy.
In order to evaluate the interest to broaden the list of pathogens included in the panel of analyses, the implication of bovine herpesvirus 4 (BoHV-4) in abortion was assessed by two different studies. In the first study, coupled serology was performed after abortion on 714 dams to identify specific seroconversion against BoHV-4. The overall seroconversion percentage in cows was of 19.5%, with a higher frequency in primiparous compared to multiparous females. In addition, the type of breed (beef cattle) and the quarter (the fourth quarter 2008 until the last quarter 2009) were significantly related to the seroconversion percentage of cows. The second study investigated the virus ability to infect the foetus. In this study, 368 cases of bovine abortions were specifically tested for BoHV-4, with both PCR on foetus tissues and ELISA on dams and foetus sera. The results showed a maternal seroprevalence of 64.7%, a foetal seroprevalence of 0.8% and a PCR prevalence in foetuses of 1.1%, demonstrating the ability of BoHV-4 to infect the foetus.

13.

Experimental feline enteric coronavirus infection reveals an aberrant infection pattern and shedding of mutational variants with impaired enterocyte tropism

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Feline coronaviruses occur as two pathotypes for which the disease-causing potential is determined by their cell tropism. Feline enteric coronavirus (FECV) is an enzootic enteropathogen that, mostly subclinically, replicates in enterocytes after oral uptake. Occasionally, not yet fully characterized mutations occur in the FECV genome, which abrogate the enterocyte tropism, but allow this mutational variant, designated feline infectious peritonitis virus (FIPV), to efficiently replicate in monocytes and cause a fatal systemic disease, known as FIP. Since crucial virological and immunological data on FECV infections are lacking, the present study investigated these missing links during experimental infection of three SPF cats with FECV strain UCD. Two cats showed mild symptoms, faecal shedding of infectious enterotropic virus from 4 dpi, a cell-associated viraemia at inconsistent time points from 5 dpi, a highly neutralising antibody response from 9 dpi, and no major abnormalities in leukocyte numbers. Faecal shedding lasted for 28-56 days, but virus shed during this stage was less enterotropic and affected by mutations. Remarkably, in the other cat, symptoms and acute shedding were not seen, but virus was detected in blood cells from 3 dpi, and shedding of non-enterotropic, mutated viruses suddenly occurred 14 dpi and lasted until 168 dpi. Neutralising antibodies arose only from 21 dpi. Leukocyte numbers were not different compared to the other cats, except for the $CD8^+$ regulatory T cells. These data indicate that FECV can infect immune cells even in the absence of intestinal replication and raise the hypothesis that the gradual adaptation to these cells can allow non-enterotropic mutants to arise.

14.

Analysis of synchronous and asynchronous *in vitro* infections with homologous murine norovirus strains

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Introduction
Noroviruses are recognised as an important cause of non-bacterial gastroenteritis in humans. Molecular mechanisms driving norovirus evolution are accumulation of point mutations and recombination. Despite numerous reports of recombination in the field, the phenomenon has been shown to be not easily reproducible in laboratory conditions. Using the murine norovirus as a model, this study aimed at qualitative and quantitative assessment of virus progenies generated by the *in vitro* use of different parameters of co- and superinfection, prerequisites for recombination events.

Materials and Methods
Cultured mouse leukaemic monocyte-macrophage cells (RAW 264.7) were infected synchronously or asynchronously with two homologous murine noroviruses (primary infection: WU20; superinfection: MNV1-CW1), using different multiplicities of infection (WU20: constant MOI of 1; CW1: MOI 0.1; 1; 10) and different times of infection (simultaneous co-infection or delays of superinfection: 30 minutes to 24 hours).

Supernatants were collected at 24 hours post co- or superinfection and were quantitatively analysed on the 5' end of the genome to differentiate between the two parental strains. Relative quantities for genomic copies of both parental strains within the total viral population 24 hours post infection were thus established.

The supernatants were subsequently inoculated onto cell monolayers and clones for each condition were selected, plaque purified and amplified. A discriminative RT-qPCR, targeting either the 3' or 5'end of the genome, was performed on each resulting progeny and allowed distinction between parental origins for estimation of infectious progeny ratios and putative recombinants.

Putative recombinants were characterised by sequencing reactions targeting the typical recombination breakpoint.

Results and Discussion
Expected ratios of genomic copies of the parental strains, depending on relative MOI and superinfection time, were rarely observed within the total viral progenies 24 hours post infection. Rather, a predominance of WU20 over CW1 was notable at all conditions of superinfection and was especially marked for longer delays between initial infection and superinfection.
This suggests a possible superinfection exclusion mechanism of the primary strain towards the superinfecting one.

For the first two infection ratios (WU20/CW1: MOI 1/0.1 and 1/1), the above observation was mirrored in the results of the quantification of infectious virus progenies on the 3' and 5' ends of the genome, in that the WU20 fraction predominated.

With regard to the ratio of WU20/CW1: MOI 1/10 however, a discrepancy to prior observations was clear. While for genomic copies a ratio of 50/50 for both parental strains was seen in the first two time points, values for CW1 now approached 90% of the population of infectious viruses.
This suggests a possbile dominance of CW1 over WU20, putatively mediated by the impact of non-infectious defective particles within the WU20 population.

Three detected putative recombination events could not be confirmed via sequencing of the main recombination breakpoint. These results confirm the apparent discrepancy which has been reported between the frequency of recombination in noroviruses in the field as opposed to the rare occurrence in laboratory conditions. Together, the results provide important data and suggest further hypotheses on recombination in the genus *Norovirus*.

15.

Simultaneously mapping local epidemics, pathogen transcriptional profile and host immune response in infants with acute respiratory infections

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Respiratory tract infections (RTIs) present a significant morbidity, posing a burden in the healthcare system. Although RTIs are most commonly caused by virus, most patients are treated with antibiotics strengthening the need for improved diagnostic methods. We aimed to quantify viral and bacterial RNA signatures using nCounter® in nasopharyngeal aspirates (NPA) obtained from 65 patients with RTI. Respiratory syncytial virus (RSVA and B), the most frequent viral pathogens, were studied in detail in a large cohort of 560 children with ARI.
This prospective cross-sectional study enrolled children (6-23-months-old) with respiratory infection for ≤7 days, from September 2009 to October 2013, in Salvador, Brazil. Upon recruitment, demographic, clinical data and NPA were collected. nCounter digital quantification was performed using a custom designed codeset for the hybridization of viral, bacterial and human targets. A multiplex quantitative RT-PCR with a subgroup-specific primer and probeset for RSVA and RSVB was used for validation. nCounter® as a diagnostic method was tested in randomized samples (n=61) of ARI patients. We detected RNA viruses PIV1-3 and RSVA-B in 21%, hMPV in 5%, BoV, CoV, FluA in 3% and, Rhinovirus) in 2% of samples, respectively. DNA virus (AV) was detected at RNA level, reflecting viral replication, in 10% of samples. Bacterial transcripts from *H. influenzae, S. pneumoniae, M. catarrhalis, M. pneumoniae and C. pneumoniae* were detected in 69, 26, 8, 3 and 2% of samples, respectively. Presence of RSV A and B was confirmed by Real Time PCR, both techniques presented good agreement. Overall, 139 (24.8%) cases had detectable RSV: RSVA was found in 74(13.2%) and RSVB in 67(12.0%) cases, including two co-infections. RSVB infection showed seasonal distribution and positive association with humidity (p=0.02). RSVA was more common among children ≥1-year (17.8% vs. 1.8%;p=0.02), while no difference was found for RSVB cases (11.5% vs. 12.2%; p=0.8).

Conclusions:
1. nCounter® proved to be a robust technique for the simultaneous detection of viral, bacterial and human immune transcripts in nasopharyngeal aspirates, even those presenting low RNA yield. 2. One quarter of the patients had RSV, with RSVA compromising more frequently children aged ≥1 year. Markedly different monthly as well as yearly patterns for RSVA and RSVB reveal independent RSV subgroup epidemics.

16.

Helminth-induced inflammation enhances cytotoxic T cell–mediated control of γ-herpesvirus infection.

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A number of studies have highlighted the impact of helminth infection on concurrent inflammatory processes such as allergic asthma but also coinfection with viruses. In particular, the tightly regulated Th2 response induced during parasitic worm infection has recently been shown to favour reactivation from latency during murid γ-herpesvirus (MuHV-4) infection in mice. In our study, we aimed to determine how acute γ-herpesvirus infection and host colonization could be affected by preliminary helminth infection. We utilised live infection with the parasitic helminth *Schistosoma mansoni (Sm)* and a *Sm* egg-induced inflammation model in the lung to generate Th2-type responses. Mice were subsequently infected intranasally with a MuHV-4-luciferase+ recombinant virus in order to track viral infection by bioluminescence. We observed that the parasite egg-induced inflammation caused a significant reduction of MuHV-4 infection levels in the lung at day 7 post-viral infection (p.i) using *in vivo* imaging and viral titration. Additionally, the egg-induced inflammation in the lung protected from the weight loss caused by the acute viral infection. Using a MuHV-4-eGFP virus, we further observed that exposure to *Sm* eggs was associated with significantly reduced numbers of alveolar macrophages supporting viral infection. At day 7 p.i., flow cytometry analysis of the lung revealed that pre-exposure to *Sm* eggs was associated with an enhanced influx of $Ly6C^+$ monocytes and significantly increased $CD8^+$ T-cells. This included increased proportions and numbers of virus-specific $CD8^+$ T cells not only the lung, but also the draining LN and spleen. Given these findings we further explored the anti-viral $CD8^+$ T cell response after *Sm* egg exposure. Firstly, depletion of $CD8^+$ cells restored viral infection to the high levels at day 7 p.i., irrespective of the exposure to *Sm* eggs. Secondly, stimulation with MuHV-4-specific $H\text{-}2^b$-restricted peptides revealed enhanced IFN-γ production and expression of the CD44 activation marker in *Sm* pre-exposed mice. Finally, we used an *in vivo* cytotoxic assay at day 7 p.i. with MuHV-4 and observed a more effective killing of virus peptide pulsed target cells in *Sm* exposed mice. These results indicate that exposure to helminths before viral infection can drive an enhanced anti-viral $CD8^+$ T cell response, which could potentially be long lived, resulting in enhanced control of persistent viral infection by γ-herpesviruses.

17.

Murid herpesvirus 4 infection protects mice from the development of pneumovirus induced immunopathologies

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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, the vaccine-enhanced disease 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. This homologous host-pathogen model was used to mimic the human respiratory syncytial virus (RSV) vaccine-enhanced disease, as observed in children during past vaccinal trials. Interestingly, using this model, we 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. Notably, we observed significant lower BALF levels of total leukocytes, eosinophils, and Th2 cytokines IL-4, IL-5 and IL-13 in MuHV-4 infected mice than in mock-infected ones. This protective impact against the immunopathology was maintained over time and required pulmonary MuHV-4 replication. Moreover, MuHV-4 infection also conferred protection in non-vaccinated mice against the lethal wild-type PVM infection. This protection was associated with an improved PVM-specific CD8 cytotoxic response that we observed in lungs of MuHV-4 imprinted mice. 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.

18.

Theiler's virus L* protein inhibits RNase L dimerization and activation by binding to the 2-5A-binding pocket of the enzyme

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We observed previously that the L* protein of Theiler's virus (picornavirus) inhibited RNase L, through direct protein-protein interaction. Interestingly, RNase L inhibition by L* exhibits species-specificity as L* inhibits mouse but not human RNase L (1). We took advantage of this species specificity to map the L* binding site on RNase L, by testing L*-mediated inhibition of a series of human-mouse RNase L chimeras. Our results suggest that L* interacts at two sites within RNase L ankyrin repeats 1 and 2. According to the recently solved structure of dimeric RNase L (2-3), our results are compatible with RNase L inhibition through both dimerization and 2-5A-binding antagonism. We therefore established a crosslinking-based RNase L dimerization assay and show that L* indeed inhibits RNase L dimerization. Next, we analyzed whether L* could interfere with 2-5A binding. Competitive binding studies by surface plasmon resonance demonstrated that L* indeed competes with 2-5A binding to mouse but not human RNase L. In conclusion, protein L* of Theiler's virus inhibits the OAS/RNase L pathway by binding to RNase L ankyrin repeats 1 and 2, thereby preventing 2-5A-mediated dimerization and activation of RNase L. L* is the first viral protein we know of that antagonizes the OAS/RNase L pathway by acting on the effector enzyme.

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19.

Single amino acid substitution in the HIV-2 gp36 ectodomain part interacting with BST-2 impairs viral release.

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1. Introduction and objectives

Tetherin (BST-2) is a host antiviral factor that retains viral particles at the cell surface and causes their endocytosis and destruction.

The HIV-2 Env protein is known to counteract tetherin during the viral replication cycle. A study demonstrated that the endocytosis motif GYRPV in the HIV-2 Env cytoplasmic tail (CT) was necessary to antagonise tetherin, but another domain in the gp36 ectodomain was involved in this antagonistic role. As this domain was not clearly defined[1, 2], we tried to identify it.

2. Methods

Since the HIV-1 Env protein does not antagonise tetherin[3], we selected 42 potential amino acids by an *in silico* comparison of the HIV-1 and HIV-2 gp 41 amino acid sequences. We used site-directed mutagenesis to introduce mutations at the sites of interest in an infectious clone. HEK293 cells were transfected with the produced clones and the generated viral particles were used to infect H9 cells (MOI=2). A RT-qPCR was performed to quantify the quantity of viral RNA released in the cell culture medium at two, three and six days post-infection. Furthermore, infected cells were treated with subtilisin (a bacterial exotoxic protease) to quantify the number of viral particles tethered at the cell surface in order to assess the antagonistic role.

3. Results

Among the 42 HIV-2 Env mutants tested, we caracterized a mutant (Env N659D) that shows a significant lack of antagonism to tetherin and of release from infected cells (Figure 1). Subtilisin treatment confirmed that this virus is more tethered at the cell surface (Figure 2).

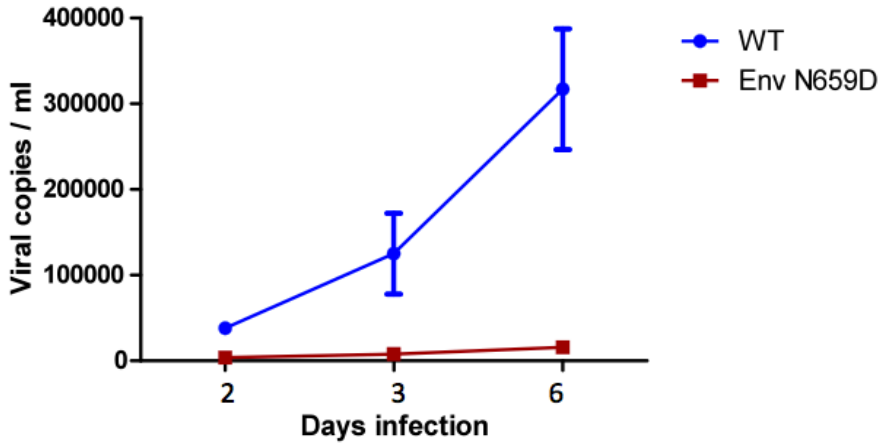

Figure 1: Quantification by RT-qPCR of viral copies produced by infected H9 cells after 2, 3 and 6 days (n=3, mean ± s.d.).

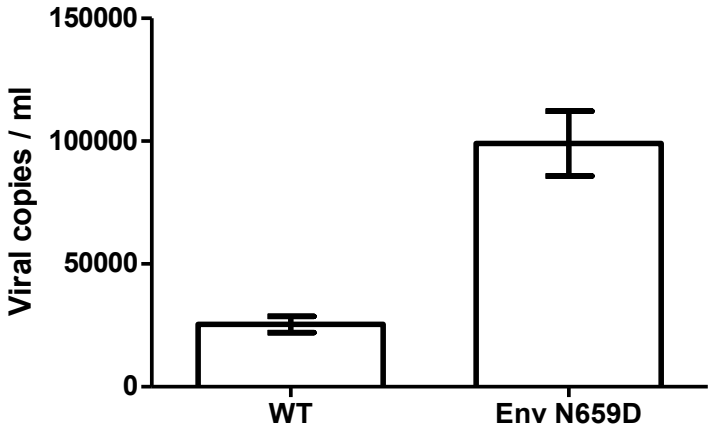

Figure 2: Quantification by RT-qPCR of viral copies released after the treatment with subtilisin (3 days post-infection; n=3, mean ± s.d.).

4. Conclusion

A single mutation in the HIV-2 gp36 ectodomain hinders the antagonistic role toward human tetherin. We demonstrated precisely that in addition to the endocytosis motif in the CT, an amino acid in the ectodomain is involved in this function.

This finding could refine our understanding of the control of HIV-2, especially the role of tetherin, and may open the way to new strategies to control the replication of HIV-1 infection.

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20.

Role of Sp1 and CTIP2 in the transcriptional regulation of Human T-Lymphotropic Virus-1 (HTLV-1)

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Human T-lymphotropic Virus 1 (HTLV-1) infects 15-20 million people worldwide and is responsible for two major diseases : adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis. 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 the transcriptional repression of viral expression. This latency represents a viral strategy to escape from the host immune system allowing tumor development. Sp1 has been demonstrated as an important regulator of eukaryotic promoter transcriptional activity. Four Sp1 sites have been previously identified in the HTLV-1 5' Long Terminal Repeat (LTR). Our *in silico* analysis of the nucleotide sequence of the HTLV-1 LTR revealed the presence of two additional potential Sp1 binding sites. We showed that the Sp1 and Sp3 transcription factors bound *in vitro* to these sites and compared the binding affinity for Sp1 of all different HTLV-1 Sp1 binding sites. Furthermore, we demonstrated Sp1 recruitment *in vivo* to the newly identified Sp1 sites. We determined in the nucleosomal context of an episomal reporter vector that the Sp1 sites interfered with both the sense and antisense LTR transcription. Interestingly, we showed that the two new Sp1 binding sites of the LTR exhibited together a repressor effect on the LTR sense transcriptional activity but had no effect on the LTR antisense activity.

Our laboratory has previously shown that the cellular transcriptional cofactor CTIP2 (COUP-TF interacting Factor 2)/Bcl11b (B-cell CLL/lymphoma 11b), involved in the development and lymphomagenesis, is recruited to the HIV-1 and p21 promoters via its association with the transcription factor Sp1, thereby silencing gene transcription through interactions with histone deacetylases and histone methyltransferases. Recently, we have reported that CTIP2 interacts with and inhibits the positive transcription elongation factor b (P-TEFb) (Cherrier *et al*., PNAS, 2015), whose deregulations are associated with various types of human malignancies. Moreover, we showed that HMGA1 (High Mobility Group A1), a protein highly expressed during embryogenesis and all aggressive human cancers, is involved in the recruitment of the P-TEFb repressor CTIP2 and/or the CTIP2-repressed P-TEFb complex to target promoters (Eilebrecht *et al*., NAR, 2014). Here, we demonstrated that CTIP2 was able to repress the TAX_{HTLV-1}-mediated transactivation of the HTLV-1 promoters. It has been reported that CTIP2 interacts with the histone acetyltransferase p300 and is involved in transcriptional activation of the IL-2 promoter in T lymphocytes. We postulated that the function of CTIP2 might be modulated by posttranslational modifications of the protein. To test this hypothesis, we evaluated posttranslational modifications of overexpressed CTIP2 protein and identified at least 5 acetylation sites. Interestingly, our recent results showed that the substitution of a single particular acetylable residue by an arginine, a non-acetylable residue, impeded the global acetylation of CTIP2. Moreover, this substitution also interfered with the ability of CTIP2 to inhibit TAX_{HTLV-1}-mediated transactivation of the HTLV-1 promoters. Finally, we demonstrated the recruitment *in vivo* of CTIP2 to the HTLV-1 LTR in a latently-infected cell line but not in a HTLV-1 productive cell line.

21.

The genome of a tortoise herpesvirus (testudinid herpesvirus 3) has a novel structure and contains a large region that is not required for replication *in vitro* or virulence *in vivo*

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Testudinid herpesvirus 3 (TeHV-3) is the causative agent of a lethal disease affecting several tortoise species. The threat that this virus poses to endangered animals is focusing efforts on characterizing its properties, in order to enable the development of prophylactic methods. We have sequenced the genomes of the two most studied TeHV-3 strains (1976 and 4295). TeHV-3 strain 1976 has a novel genome structure and is most closely related to a turtle herpesvirus, thus supporting its classification into genus *Scutavirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae*. The sequence of strain 1976 also revealed viral counterparts of cellular interleukin-10 and semaphorin, which have not been described previously in members of subfamily *Alphaherpesvirinae*. TeHV-3 strain 4295 is a mixture of three forms (m1, m2, and M), in which, in comparison to strain 1976, the genomes exhibit large, partially overlapping deletions of 12.5 to 22.4 kb. Viral subclones representing these forms were isolated by limiting dilution, and each replicated in cell culture comparably to strain 1976. With the goal of testing the potential of the three forms as attenuated vaccine candidates, strain 4295 was inoculated intranasally into Hermann's tortoises (*Testudo hermanni*). All inoculated subjects died, and PCR analyses demonstrated the ability of the m2 and M forms to spread and invade the brain. In contrast, the m1 form was detected in none of the organs tested, suggesting its potential as the basis of an attenuated vaccine candidate. Our findings represent a major step towards characterizing TeHV-3 and developing prophylactic methods against it.

22.

Epigenetic regulation of gga-microRNA-126 during lymphoproliferative disease in chicken

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Both in human and animal, some of the herpesvirus latent infections progress to cancer. Gallid herpesvirus-2 (GaHV-2), an oncogenic α-herpesvirus, modulates viral and cellular gene expression and triggers transformation of latently infected cells. This avian pathogen, responsible of the Marek's disease (MD), naturally infects chickens and provides a unique model for studying virus induced lymphoma development. This study focus on a microRNA (gga-miR-126) downregulated during the viral life cycle. Originally described as a miRNA mediating proper angiogenesis and vascular integrity, gga-miR-126 has been reported to impair cancer progression through signaling pathways that control tumor cell proliferation, migration and survival. First, in order to confirm the downregulation of gga-miR-126, it was quantified by Q-RT-PCR, in tumoral latently infected cell line (MSB-1) and nine organs of three uninfected chicken. Gga-miR-126 was undetectable in the infected cell lines while in the nine organs it was expressed. Gga-miR-126 expression was more abundant in the lung and the heart. It can be concluded that gga-miR-126 is not detectable in tumor-induced latently infected cells. Then, to characterize this low expression level, DNA methylation pattern was assessed in MSB-1 cell line and in the nine organs through Bisulfite Genomic Sequencing Assay (BGSA). Gga-miR-126 is located on chromosome 17 into the 6th intron of the cellular gene EGFL-7 (Epidermal Growth Factor Like-7). By an in silico analysis, two CpG islands were found in this gene. One CpG island (CpG1) is located in the first intron and the other one (CpG2) is located around the precursor of gga-miR-126. The DNA methylation pattern was assessed in these two CpG islands. In MSB-1 cell line, the level of DNA methylation was very high at the two CpG islands (CpG1, 85 % and CpG2, 95 %). In the nine organs, DNA methylation was also observed in CpG1 and CpG2 with a different percentage. Indeed, a lower level of methylation was observed in CpG1 (from 7 % to 31 %) while CpG2 presented a high level of methylation (from 53 % to 85 %). The repression of gga-miR-126 in latently infected cells (MSB-1) might be explained by the high percentage of methylation at the two CpG islands. In human, these two CpG islands are also present in EGLF-7 and DNA methylation was assessed at CpG1 site in normal and tumoral cells (Watanabe et al., 2012). In tumoral cells CpG1 site presented a high percentage of methylation while in normal cells a low level of DNA methylation was observed. Watanabe et al., quantified then hsa-miR-126 in tumoral and normal cells and observed that a low and a high expression were observed, respectively. These results showed that there is a relation between CpG1 site and the regulation of hsa-miR-126 in cancerous and normal cells. For now in the chicken, it was observed a low level of gga-miR-126 in tumoral cells (MSB-1). This repression of gga-miR-126 could be explained by the high level of DNA methylation in the two CpG islands (CpG1 and 2) present into EGFL-7.

23.

Role of bovine leukemia virus micro-RNAs in replication and oncogenesis

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Bovine leukemia virus (BLV), a retrovirus closely related to the human T-lymphotropic virus type 1 (HTLV-1), induces lymphocytosis and B-cell leukemia/lymphoma in ruminants. Although the provirus undergoes transcriptional silencing during transformation, BLV-infected tumor cells massively express viral microRNAs. To understand the mechanisms involved, a provirus isogenic to a wild type molecular clone but devoid of microRNAs was inoculated into calves. High-throughput RNA-sequencing and bioinformatic analyzes indicated that BLV microRNAs modulate expression of genes involved in B-cell signaling, cancer and immunity (GZMA, FOS, PPT1, ANXA1, MAP2K1 and PIK3CG). Direct and indirect interactions within these regulatory pathways were validated by luciferase reporter assays. In vivo, the deletion of BLV microRNAs reduced proviral loads in bovines, indicating their role in viral replication and persistence. Furthermore, oncogenesis was abolished in the ovine experimental host. These observations thus show that BLV microRNAs affect key signaling pathways, promote viral replication and drive leukemogenesis.

24.

Characterisation of oral murine adenovirus type 1 infection in mouse and evaluation of the protection induced against a respiratory homologous infection

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Oral vaccination offers many immunological and practical advantages. Nevertheless, oral immunization may be hampered by tolerance mechanisms. A solution to this problem could lie in the use of vectors such as viral vectors. Since the 1970s, alive, orally administered adenovirus serotype 4 and 7 vaccines are effectively used to protect United States military personnel from severe respiratory diseases caused by the same viruses. Replication-competent adenoviruses appear therefore as promising vectors for the development of oral vaccines. Until now, as human adenoviruses replicate efficiently only in a highly restricted host range, researches on this topic have suffered from the lack of reliable animal models. In this study, we used Murine adenovirus type 1 (MAV-1) to characterize adenovirus oral infection in mice. Briefly, while we did not observe any clinical signs associated with the oral administration of the virus, viral DNA was detected by qPCR in various organs, showing that the virus efficiently infects mice by the oral route. This infection was associated with an increase in MAV-1 specific and neutralizing antibodies over time. We finally evaluated the protection induced by MAV-1 oral infection against a respiratory challenge with the same virus. Clinical observations and histological analyses showed that orally immunized mice were protected against the severe symptoms observed after intranasal infection of naive mice. Altogether, these results show that MAV-1 offers a reliable model for oral vaccination based on replicative adenoviruses. This model provides a precious tool for studying the potential of orally administered adenoviruses as vaccine platforms.

25.

Investigation of a possible link between vaccination and the 2010 Sheep pox epizootic in Morocco

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Objective: In 2010 sheep pox virus (SPPV) re-appeared in Morocco, following previous vaccination campaigns, causing a nodular clinical form previously not observed in Morocco. Furthermore, initial reports, citing similarity in nucleotide sequence between the Moroccan vaccine strain and field isolates, warranted a more in depth analysis of this epizootic. It was the purpose of this study to (1) look more closely into the epizootic of 2010; (2) investigate a possible link between the Moroccan vaccine and the virus isolated from the field by exploring sequence similarity in different regions of the genome and by developing PCR methods to differentiate between vaccine and wild-type virus.
Methods: Samples collected from 19 flocks located within four provinces the eastern region of Morocco during the 2010 Outbreak were analysed using a real-time PCR panel and an in-house monolayer Elisa. Isolates from different geographic regions were phylogenetically analysed and compared to each other and to the vaccine used in the region. DIVA PCRs were developed to analyse a possible link between the isolates and vaccine.
Results: Aside from scab material, blood was the sample type which most frequently gave a positive result (98% positive) followed by buccal and ocular swabs, 93% and 91% positive, respectively. However, most variability was seen in blood samples when using the Haegeman PCR panel, ranging from 64%, 69% to 85% positivity. Seroconversion for Capx was detected in 80.5% of the animals and in each flock.
Sequence analysis of two genomic regions showed that all isolates obtained from four the provinces of Eastern Morocco were identical and were clearly different from the Moroccan vaccine strain. Using two newly developed DIVA PCRs no trace of wild type SPPV was found in the vaccine and no trace of the vaccine was found in the sampled animals.
Conclusion: Ocular swabs were found to be a useful sample type as a detection rate of 91% was obtained. For rectal and nasal swabs the detection rates were noticeably lower probably due to a greater sensitivity to the timing of sampling relative to the course of infection. Buccal swabs (detection rate of 93%) were found to be an interesting alternative with the added advantage of being easier to take than ocular swabbing. The PCR-panel detection rate in blood was found to be 98%. However, this sample type may be less suited as the individual PCR detection rates of the PCR-panel were more variable.
Based on the sequences data, it can be stated that a single SPPV strain was responsible for the 2010 epizootic. In addition, no evidence was found linking the vaccine (vaccine strain or presence of wild type virus) directly to the epizootic. However, further analysis is needed to clarify the epidemiological picture in relation to recombination, re-introduction or re-emergence. The two newly developed PCRs, able to differentiate between the RM-65 vaccine strain and wild type SPPV, can be a useful tool in future epidemiological investigations during vaccination programs.

26.

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

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RSV is responsible for 64 million infections/year and severe bronchiolitis in children and elderly. Besides this acute pathology, RSV is also linked to chronic pulmonary problems, like asthma and recurrent wheezing. There is a clear link between the pathology and the immune response, so various research groups have studied the possible role of macrophages, which are prominent cells of the lung immune system and appear to be permissive for RSV. Published results however are often contradictory. A viable explanation is that diverse types of macrophages cell lines were used which may exhibit different sensitivities towards RSV infection. The aim of this study is to evaluate the susceptibility of different macrophage cell lines for RSV.
The murine macrophage cell lines MH-S, RAW 264.7 and J774 were infected with the RSV strain A2 and fixed at 2 and 24h p.i. Cells were permeabilized and RSV antigens were stained with a polyclonal anti-RSV serum followed by an AF488-labelled conjugate. Both MH-S (2%) and RAW 264.7 (0,4%) cells showed clear staining of RSV-antigens in the cytoplasm 24h p.i. The number of infected cells was low, yet significant since the staining was more intense compared to the staining at 2h p.i., indicating that new RSV-antigens were synthesized and that cells were indeed infected. J774 cells showed no positive signal of RSV-antigens.
A double staining showed that RAW 264.7 cells, in contrast to MH-S cells, express no RSV-antigens on the surface. This suggests an abortive RSV-infection in RAW 264.7 cells. This was confirmed by inoculating HEp-2 cells with supernatants of infected cells, collected 24 and 72h p.i. The percentage of infected HEp-2 cells increased from 1,5 to 5% when 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, the RSV-infection with the A2 strain varies among macrophage cell lines and only MH-S appear to be fully susceptible, yet in a low percentage of the cells.

27.

Comparison of clonal integration site frequency and replication competent virus by viral outgrowth and TILDA in patients on long term ART

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Background: The latent HIV-1 reservoir remains one of the major obstacles to cure HIV. To monitor HIV cure strategies, a validated biomarker is needed that can evaluate the reservoir over time *in vivo*. Consequently, numerous assays are now being investigated to estimate the size of the replication competent provirus.

Methods: A comprehensive study was designed to evaluate and compare potential HIV-1 reservoir biomarkers. A cohort of 25 ART treated patients was sampled in which plasma viral load (<50 cp/ml) was suppressed for median of 7 years (5-11). Total, integrated HIV-1 DNA and unspliced (us-) HIV-1 RNA were quantified in peripheral blood. A quantitative viral outgrowth assay (qVOA) was performed. Subsequently, a nested substudy of 10 patients was set-up to analyse HIV-1 integration sites and tat/rev induced limiting dilutions assay (TILDA). The selected patients had low level of total HIV-1 DNA (<250 cp/10^6PBMCs) or high level of qVOA (> 2 IUPM). The percentage of integration site clonality was estimated based on results from the integration site analysis, and the frequency of cells with inducible HIV RNA transcription were estimated using maximum likelihood method.

Results: Integrated and total HIV-1 DNA were detected in all patients, and both measures correlated well (p=0.002, R^2=0.85). Replication-competent virus was detected in 80% of patients by the qVOA and this correlated with integrated and total HIV-1 DNA (p=0.05, R^2=0.44; p=0.019, R^2=0.54; respectively). In total 317 integration sites were analysed. A wide range of percentage of clonality (25.0-91.4%) was observed between patients. The majority of patients had an average of 30% clonality, which on average comprised of 3.4 predominant clonal integration sites per patient. Patients with higher clonal diversity (≥ 6 clones) had higher estimate for TILDA (frequency of cells with inducible msRNA > 200 cells /10^6 PBMCs). Interestingly, one patient had an extremely high clonality (>90%), which was represented by a single clone. This patient also had no inducible virus in both VOA and TILDA, suggesting that the clonal provirus in this patient is replication incompetent. The single predominant clone is thought to be associated with the extremely CD4 nadir count in this patient (3 cells/ul.)

Conclusion: We observed a good correlation between VOA and integrated and total HIV-1 DNA. Integration site sequencing revealed that most of the ART treated patients in this study had an HIV DNA reservoir consisting of 30% clonally expanded cells. These levels of clonal provirus did not affect the amount of inducible virus, except for the one patient with >90% clonality.

28.

An In-Depth Comparison of Latency-Reversing Agent Combinations in Various *in vitro* and *ex vivo* HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression.

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The persistence of latently infected cells in patients under combinatory antiretroviral therapy (cART) is a major hurdle to HIV-1 eradication. Strategies to purge these reservoirs are needed and activation of viral gene expression in latently infected cells is one promising strategy. Bromodomain and Extraterminal (BET) bromodomain inhibitors (BETi) are compounds able to reactivate latent proviruses in a positive transcription elongation factor b (P-TEFb)-dependent manner. In this study, we tested the reactivation potential of protein kinase C (PKC) agonists (prostratin, bryostatin-1 and ingenol-B), which are known to activate NF-κB signaling pathway as well as P-TEFb, used alone or in combination with P-TEFb-releasing agents (HMBA and BETi (JQ1, I-BET, I-BET151)). Using *in vitro* HIV-1 post-integration latency model cell lines of T-lymphoid and myeloid lineages, we demonstrated that PKC agonists and P-TEFb-releasing agents alone acted as potent latency-reversing agents (LRAs) and that their combinations led to synergistic activation of HIV-1 expression at the

viral mRNA and protein levels. Mechanistically, combined treatments led to higher activations of P-TEFb and NF-κB than the corresponding individual drug treatments.
Importantly, we observed in *ex vivo* cultures of $CD8^+$-depleted PBMCs from 35 cART-treated HIV-1^+ aviremic patients that the percentage of reactivated cultures following combinatory bryostatin-1+JQ1 treatment was identical to the percentage observed with anti-CD3+anti-CD28 antibodies positive control stimulation. Remarkably, in *ex vivo* cultures of resting $CD4^+$ T cells isolated from 15 HIV-1^+ cART-treated aviremic patients, the combinations bryostatin-1+JQ1 and ingenol-B+JQ1 released infectious viruses to levels similar to that obtained with the positive control stimulation. The potent effects of these two combination treatments were already detected 24 hours post-stimulation. These results constitute the first demonstration of LRA combinations exhibiting such a potent effect and represent a proof-of-concept for the co-administration of two different types of LRAs as a potential strategy to reduce the size of the latent HIV-1 reservoirs.

29.

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

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Gammaherpesviruses are important human and animal pathogens. Despite they display the classical architecture of herpesviruses, the function of the most of their structural proteins is still poorly defined. This is especially true for the tegument proteins. Interestingly, a potential role in immune evasion has recently been proposed for the tegument protein encoded by ORF63. However, this study did not involve the construction of an ORF63 knockout strain and the significance of these results remains unknown. In this project, we wanted therefore to better define the importance of ORF63 in the lifecycle of Murid Herpesvirus 4 (MuHV-4). We showed that a lack of ORF63 was associated with a severe viral growth deficit both in vitro and in vivo. The latter deficit was mainly associated with a defect of replication in the lung but did not appear to be due to a reduced ability to establish the latency. On a functional point of view, inhibition of caspase-1 or 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 MuHV-4 ORF63 deficient mutants failed to address most of their capsids to the nucleus during entry, suggesting that ORF63 plays a role in capsid movement along the microtubule network. In the future, ORF63 could therefore be considered as a target to block gammaherpesvirus infection at a very early stage.

30.

Equine herpesvirus type 1 (EHV-1) enhances viral replication in CD172a$^+$ monocytic cells upon adhesion to endothelial cells

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Abstract

Equine herpesvirus type 1 (EHV-1) is a main cause of respiratory disease, abortion and encephalomyelopathy in horses. Monocytic cells (CD172a$^+$) are the main carrier cells of EHV-1 during primary infection and are proposed to serve as a 'Trojan horse' to facilitate the dissemination of EHV-1 to target organs. However, the mechanism by which EHV-1 is transferred from CD172a$^+$ cells to endothelial cells (EC) remains unclear. The aim of this study was to investigate EHV-1 transmission between these two cell types. We hypothesized that EHV-1 employs specific strategies to promote the adhesion of infected CD172a$^+$ cells to EC to facilitate EHV-1 spread. Here we demonstrated that EHV-1 infection of CD172a$^+$ cells resulted in a 3 to 5-fold increase in adhesion to EC. Antibody-blocking experiments indicated that $\alpha_4\beta_1$, $\alpha_L\beta_2$ and $\alpha_V\beta_3$ integrins mediated adhesion of infected CD172a$^+$ cells to EC. We showed that integrin-mediated PI(3)K and ERK/MAPK signaling pathways were involved in EHV-1-induced CD172a$^+$ cell adhesion at early time of infection. EHV-1 replication was enhanced in adherent CD172a$^+$ cells, which correlates with the production of TNF-α. In the presence of neutralizing antibodies, approximately 20% of infected CD172a$^+$ cells transferred cytoplasmic material to uninfected EC and 0.01% of infected CD172a$^+$ cells transmitted infectious virus to neighbouring cells. Our results demonstrated that EHV-1 infection induces adhesion of CD172a$^+$ cells to EC, which enhances viral replication, but that transfer of viral material from CD172a$^+$ cells to EC is a very specific and rare event. These findings give new insights in the complex pathogenesis of EHV1.

31.

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

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Antibody-induced internalization is an important process that may modulate the surface expression of viral glycoproteins. It has been described for several viruses, including herpesviruses and measles virus. Also for respiratory syncytial virus (RSV), it was 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. Further research was performed to determine whether only one or both RSV major surface proteins F and G undergo internalization. RSV-infected cells and cells transfected with plasmids encoding RSV F or G were incubated with polyclonal or monoclonal RSV-specific antibodies. Both strategies resulted in a clear uptake of RSV antigen-antibody complexes in a time-dependent manner. The process was rapid and reached a maximum after 90 minutes. In addition, flow cytometric analysis after induction of internalization showed a clear reduction in surface expressed RSV antigens. By using infected cells with biotin-labelled surface proteins, a clear difference was observed between internalization of the RSV F protein induced by antibodies and spontaneous endocytosis, confirming that antibody binding triggers internalization.
Of the RSV surface proteins, RSV F is the most conserved and the main target of neutralizing antibodies, including Palivizumab which is the only approved immunoprophylaxis. Current research into the development of new immunoprophylaxis and vaccines is mainly focused on the RSV F protein. Inhibitors of endocytic mechanisms and dominant-negative proteins were used to identify the mechanism through which RSV F antigen-antibody complexes are internalized. The findings of these experiments suggest a clathrin-dependent mechanism. Internalization was induced by different neutralizing pre- and/or postfusion RSV F specific antibodies, including Palivizumab, indicating that this process is epitope-independent. For herpesviruses, antibody-induced internalization was shown to interfere with antibody-dependent complement-mediated lysis. It remains to be determined whether this process for RSV can act as an immune evasion mechanism and play a rol in the partial failure of RSV antibody responses and immunoprophylaxis. In this context, experiments are ongoing to identify the responsible endocytic motifs in the cytosolic domains. By using a BAC-based RSV rescue system, the development of recombinant viruses deficient in RSV F internalization could be achieved.

32.

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

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Carbohydrates play major roles in host-virus interactions. It is therefore not surprising that, during co-evolution with their hosts, viruses have developed sophisticated mechanisms to hijack to their profit different pathways of glycan synthesis. Thus, the Bo17 gene of Bovine herpesvirus 4 (BoHV-4) encodes a homologue of the cellular core 2 β-1,6-N-acetylglucosaminyltransferase-mucin type (C2GnT-M) which is a key player for the synthesis of complex O-glycans. Surprisingly, we show in this study that, as opposed to what is observed for the cellular enzyme, two different messenger RNAs are encoded by the Bo17 gene of all available BoHV-4 strains. While the first one corresponds to the entire coding sequence of the Bo17 gene, the second results from the splicing of a 138 bp intron encoding critical residues of the enzyme. Antibodies generated against the Bo17 C-terminus showed that the two forms of Bo17 are expressed in BoHV-4 infected cells, but enzymatic assays revealed that the spliced form is not active. In order to reveal the function of these two forms, we then generated recombinant strains expressing only the long or the short form of Bo17 and showed by glycomic analyses that BoHV-4 uses alternative splicing to markedly regulate the cellular core 2 branching activity of infected cells. We therefore postulate that the relative abundance of active/inactive forms of pBo17 in Golgi oligomeric complexes may define the global level of C2GnT-M activity in the infected cell. These results suggest the existence of new mechanisms to regulate the activity of glycosyltransferases from the Golgi apparatus.

33.

Feline herpesvirus 1 replication in ocular and respiratory mucosa explant model

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Objective: Feline herpesvirus 1 (FeHV-1) is one of the major viral pathogens involved in rhinotracheitis and ocular diseases in cats. Little is known about how the virus replicates in and invades the mucosae of the respiratory tract and eyes, resulting in lesions. In the present study, viral behavior during the early stage of infection was examined in the respiratory and ocular mucosae, the portals of entry of FeHV-1 by using *ex vivo* explants.

Methods: Three FeHV-1 seronegative cats without ocular or respiratory problems were used in this study. Equal pieces ($25mm^2$) of corneal, conjunctival and tracheal mucosa explants were maintained in an air-liquid culture at 37 °C and 5% CO_2, and its viability was examined during 96 h of cultivation. All tracheal tissues were observed under a stereomicroscope for cilia beating, and only tracheal tissues in which cilia beating was present all over the explants were used. Corneal, conjunctival and tracheal explants were inoculated with the FeHV-1 C-27 strain at 24 hours after cultivation, and sampled at 0, 24, 48 and 72 hours post inoculation (hpi). Viral antigen positive cells were stained by a direct immunofluorescence staining and series of stained cryosections were acquired with a Leica TCS SP2 confocal microscope. A quantitative morphological analysis of viral replication and invasion in the mucosae was performed by measuring plaque latitudes and penetration depths underneath the basement membrane (BM).

Results: The explants of three cats were maintained in an air-liquid culture up to 96 hours with a little decrease in tissue viability. After inoculation with FeHV-1(C27), FeHV-1 replicates in conjunctival and tracheal mucosae in a plaque wise manner but no infection was observed in corneal epithelium. The plaques were observed from 24 hpi and plaque latitude increased over time. The viral plaque latitudes increased over time at a similar speed in trachea and conjunctiva. FeHV-1 penetrated the BM in conjunctival and tracheal explants between 24 and 48 hpi, and most plaques crossed the BM at 72 hpi. FeHV-1 invaded the lamina propria more efficiently in tracheal tissues than in conjunctival tissues.

Conclusion: The feline ocular and respiratory explants were suitable to study the early events of the pathogenesis of FeHV-1 infections. Our study indicates that FeHV-1 has a better capacity to invade the respiratory mucosa than the conjunctival mucosa, and prefers the conjunctiva but not the cornea as a portal of entry during ocular infection.

34.

A gammaherpesvirus infection protects from allergic asthma development

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The innate immune system has a key role in initiating and in regulating polarization of immune responses. Although progress has been made to understand the role of innate immunity in inducing protective responses against pathogens, little is known about how these infections modulate subsequent heterologous immune responses. Interestingly, the hygiene hypothesis postulates that exposure to some microbes early in life prevents the later development of allergic diseases. In particular, while some viral infections exacerbate asthma lesions, others could induce protection against allergic sensitization. Here, we investigated how the highly prevalent gammaherpesvirus infections affect the subsequent development of allergic asthma. Our results demonstrate that infection by Murid herpesvirus 4 (MuHV-4) inhibits the development of House Dust Mites (HDM)-induced airway allergy in mice by modulating the function of innate immune cells involved in sensitization against HDM. Thus, while MuHV-4 infection does not influence HDM uptake by dendritic cells (DCs) nor their migration to the draining lymph nodes, it significantly decreases MHCII expression on migratory DCs and affects their capacity to prime a HDM-specific Th2 response. Initial polarization of the lung immune responses results of complex and still incompletely defined interactions between innate immune cells. Among these cells, alveolar macrophages (AM) could play a prominent role as they represent the most abundant leucocytes found in alveoli and distal airspaces. In our model, we observed by FACS analysis some strong phenotypic modifications induced on AMs following infection such as an increased expression of MHCII and decreased expression of siglec F. Beside these changes, differences in the AM gene expression profile were revealed by RNA sequencing and could be linked to the reduced capacity of DCs to initiate a Th2 response against HDM. Indeed, adoptive transfer of bone marrow-derived DCs (BMDC), co-cultivated with AMs from MuHV-4 infected mice demonstrated the loss of ability of these BMDC to induce HDM allergic sensitisation. Altogether, our results demonstrate the strong imprinting of a gammaherpesvirus infection on lung innate immune cells and the subsequent consequences on asthma development. In the future, this model could allow us to highlight key steps of the Th2 polarization against respiratory allergens and to develop new strategies against allergic asthma.

35.

Enhanced pig-to-pig transmission of a reassortant H9N2 influenza virus containing 2009 pandemic H1N1 internal genes by serial passaging in pigs

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Avian influenza viruses of H9N2 subtype are endemic in poultry in Asia and the Middle East. Since 1998, they have caused sporadic dead-end infections in humans and swine. Along with avian H7 and H5 influenza viruses, H9N2 is in the top-3 of the World Health Organization's list of pandemic influenza virus candidates. On the other hand, the 2009 pandemic H1N1 (pH1N1) virus originated from and has become endemic in swine. Its internal gene cassette has extensively reassorted with established swine influenza viruses all over the world and also appears to be highly compatible with the surface protein genes of H9N2. This finding has raised concerns about the possible emergence in swine of H9N2 reassortants with public health implications. Moreover, such reassortant viruses showed increased replication and transmissibility in the mouse and ferret model of influenza. The few studies in pigs also suggested improved fitness of the reassortants as compared to the parental H9N2 virus. We aimed to evaluate if a reassortant H9N2 virus could become better adapted to pigs by serial passaging. To investigate this we performed pig transmission experiments with four different viruses: a reassortant virus containing A/Quail/Hong Kong/G1/97 (H9N2) surface genes and A/California/04/09 (pH1N1) internal genes; another reassortant virus with the same gene constellation that had undergone seven passages in pigs and both parental viruses, H9N2 and pH1N1. Replication efficiency in the porcine lower respiratory tract was minimal for the reassortant H9N2 virus, higher for the wholly avian H9N2 virus and greatest for the pig-passaged H9N2 reassortant, with 0, 55 and 93% of the lower respiratory tract samples testing positive for the respective viruses. In pig transmission studies, pH1N1 was excreted in high amounts by all three directly inoculated pigs and six out of six direct contact pigs (mean area under the curve (AUC) of 19,7 for the contact pigs). All animals inoculated with the different H9N2 viruses excreted virus but nasal swab virus titers were highly variable. In contrast, none of the contact pigs shed high amounts of the parental H9N2 or the original H9N2 reassortant virus (means AUC 3,0 and 4,7, respectively), pointing to inefficient virus transmission. Remarkably, the pig-passaged reassortant virus was excreted in high titers by four out of six contact animals, with a mean AUC of 16,1. To identify mutations that appeared during serial passaging and that may be involved in enhanced transmission we are currently performing genetic analysis of the reassortant viruses. Our data confirm that the pH1N1 internal gene cassette enhances avian H9N2 transmissibility and support the hypothesis that pigs could be suitable intermediate host for the adaptation of reassortant H9N2 virus to mammals. Our pig serial-passaging and transmission model hold potential to study the adaptation of avian influenza viruses to mammals.

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36.

A broadly neutralizing monoclonal antibody targeting hepatitis C virus prevents viral infection *in vitro* and in uPA/SCID mice with humanized liver

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Liver transplantation is the only option for patients with hepatitis C virus (HCV) induced end-stage liver diseases. However, re-infection of the newly grafted liver occurs immediately after transplantation. Although the recent spectacular improvement in success rate of HCV treatment using direct-acting antivirals, patients with severe liver disease and immune compromised liver transplant patients remain difficult to treat. Instead of trying to treat HCV before or after liver transplantation, an alternative approach could be to specifically interfere with the viral entry step and thereby prevent infection of the donor liver. This could be done by using antibodies that neutralize the circulating virus and/or targeting the viral receptors. We and others have previously reported that antibodies against CD81, SR-BI and claudin-1 could protect Huh7 cells and humanized mice from HCV infection. In the present study, we developed a human monoclonal antibody, designated 2A5, which targets the HCV envelope protein. The antibody was isolated from a chronically infected HCV genotype 1b patient. The ability of this antibody to inhibit HCV entry was assessed *in vitro* using the HCV pseudoparticle (HCVpp) and HCV cell culture (HCVcc) systems. In addition, it was also evaluated *in vivo* using the human liver chimeric urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mouse model. Results of HCVpp neutralization showed the ability of this antibody to efficiently prevent viral entry of (sub-)genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6a. The broad activity of mAb-2A5 was also corroborated using the HCVcc system where high level neutralization was observed against genotype 1a, 1b, 2a, 4a, 5a, 6a, 7a. HCVcc of genotype 3a (S52/JFH) seemed relatively resistant to neutralization. *In vivo* neutralization experiments using the human liver chimeric mice showed that a single intraperitoneal injection of mAb-2A5 three days before viral challenge resulted in complete protection in case of mH77 (gt1a) and mED43 (gt 4a), while two out of three HK6a (gt 6a) inoculated mice were protected. Although only two out of six mice inoculated with the gt1b strain mP05 were completely protected from infection, a considerable delay in viral kinetics was observed in two other 2A5-treated mice. mP05 was previously reported to be more resistant to neutralizing antibodies and to have increased entry capacities. In conclusion, mAb-2A5 shows potent anti-HCV neutralizing activity both *in vitro* and *in vivo*. mAb-2A5 could hence be a valuable tool to prevent HCV reinfection in HCV liver transplant patients.

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37.

PCR detectability and stability of pseudorabies virus in porcine oral fluid

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Aujeszky's disease (AD) is a severe and notifiable swine disease with substantial economic consequences for the pig industry. Despite its eradication in domestic pigs in several European countries, the threat for a reintroduction remains since the virus is still circulating within the wild boar population. To maintain an AD negative status, domestic pigs are routinely monitored. Oral fluid has recently been described as an alternative diagnostic matrix for swabs and serum for virological and serological monitoring of several porcine viruses, e.g. PRRSV and swine influenza (SIV). Since it allows sample collection at pen-level, we evaluated the use of oral fluid as a new specimen for monitoring of Aujeszky's disease.

First, an in vitro study was conducted to evaluate the effect of conservation time and temperature of oral fluid on PRV detectability by qPCR and virus isolation. Spiking PRV to a high and low concentration in oral fluid showed that no significant loss in qPCR detectability occurred over a 7 days conservation period when stored at 4°C. When conserved at room temperature, a moderate 10 to 20 fold decrease in detectable PRV DNA was observed over the 7 day period. Interestingly, also virus infectivity remained largely unaffected when conserved in saliva at 4°C since PRV could be isolated till 7 days post spiking at the highest concentration. Our results indicate that PRV is more stable in oral fluid than other porcine viruses like PRRSV and SIV. Secondly, two commercial blocking ELISA kits (Prionics PRV-gB and Idexx PRV-gE) were modified into indirect ELISAs in order to detect the presence of anti-PRV antibodies in oral fluid.

Afterwards, an in vivo study with two pens containing each 5 15-weeks old pigs was performed to compare these newly developed tests to routinely used diagnostics in serum and swabs. All pigs were intranasally inoculated with 10^5 $TCID_{50}$ of the virulent NIA3 strain. Pigs in pen A were naive to PRV at the moment of inoculation while pigs in pen B had been vaccinated twice with a commercial PRV vaccine (Geskypur) at 5 and 2 weeks before intranasal inoculation. Swabs and serum were collected from all individual animals at different time points while oral fluid was collected at pen level.

For both experimental groups, the comparison showed that swabs were more suitable to detect PRV shedding by qPCR at 1 and 2 dpi. Starting from 3 to 11 dpi, PRV detection was equally efficient in oral fluid and in swabs. At 14 dpi, results indicated that PRV might be longer detectable in oral fluid than in swabs. Besides the viral DNA detection, the presence of PRV specific antibodies was evaluated in serum and oral fluid collected from both experimental groups. PRV specific antibodies were detected in both matrices with a similar sensitivity. However, the capacity of differentiating infected from vaccinated animals (DIVA strategy) was no longer possible in the adapted ELISA set-up.

This study shows the usefulness of oral fluids for virological and serological PRV detection and underlines the stability of PRV DNA in this diagnostic sample.

38.

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

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The innate immune system relies on numerous molecules that act as sensors of nucleic acids. Nucleic acids are detected based on their structure, subcellular localization or sequence. Zalpha domains are 66 amino acid long domains which bind to left-handed dsDNA or dsRNA (Z-DNA/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. Carp, like all cypriniform fish, encodes PKZ, a paralogue of the dsRNA-dependent protein kinase (PKR) expressed by all vertebrates. While PKR possesses dsRNA binding domains, PKZ has Z-DNA/Z-RNA binding domains. Both proteins, once activated by binding to the appropriate nucleic acids, phosphorylate eIF-2α thereby blocking protein synthesis. Recently, it was demonstrated that ORF112 of Cyprinid herpesvirus 3 (CyHV-3) encodes a Zalpha domain over-competing the binding of PKZ to Z-DNA. In the present study we investigated the expression of ORF112 in CyHV-3 infected cells and determined whether this gene is essential to viral growth *in vitro*. Western blot and immunofluorescent staining demonstrated that ORF112 is expressed as an abundant and early protein localizing both in the nucleus and the cytosol of infected cells. In the cytoplasm, ORF112 was shown to co-localize with stress granules and ds-RNA. Finally, production of recombinant CyHV-3 BAC plasmids demonstrated that ORF112 is a gene essential to viral growth *in vitro*. Altogether, these results support the interest of the CyHV-3 – carp model as a unique model to test the roles in anti-viral innate immunity of Zalpha domain proteins detecting unusual nucleic acid conformations.

39.

Immunization of African indigenous pigs with attenuated genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of genotype I.

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Abstract

The attenuated African swine fever virus genotype I strain OURT88/3 has previously been shown to induce protection of European breeds of domestic pigs against challenge with virulent isolates. To determine whether protective immune responses could also be induced in indigenous breeds of pigs from the Kinshassa region in Democratic Republic of Congo, we immunized a group of eight pigs with OURT88/3 strain and challenged the pigs 3 weeks later with virulent genotype I strain OURT88/1. Four of the pigs were protected against challenge. Three of the eight pigs died from African swine fever virus and a fourth from an unknown cause. The remaining four pigs all survived challenge with a recent virulent genotype I strain from the Democratic Republic of Congo, DRC 085/10. Control groups of non-immune pigs challenged with OURT88/1 or DRC 085/10 developed signs of acute ASFV as expected and had high levels of virus genome in blood.

40.

Genome sequencing of alcelaphine herpesvirus 1 C500 BAC clone and WC11 attenuated strain identified duplicated and deleted regions involving ORF50, A6, A7 and A8.

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Alcelaphine herpesvirus 1 (AlHV-1) is a gammaherpesvirus carried asymptomatically by wildebeest. When it is cross-transmitted to other ruminants, they develop a lymphoproliferative disease named malignant catarrhal fever (MCF). Though the expression of the latency-associated protein encoded by ORF73 has been shown to be essential for MCF induction, the pathogenic mechanisms inducing the lymphoproliferation are yet to be identified. The AlHV-1 genome consists in a long unique region (LUR) containing 73 ORFs flanked by polyrepetitive DNA units (prDNA). AlHV-1 can be cultured *in vitro* and rearrangements of the AlHV-1 genome have been described after multiple passages in cell culture and are associated with increased cell-free viral particles and loss of pathogenicity. In this study, we sequenced the genomes of the infectious and pathogenic AlHV-1 C500 BAC clone as well as the high-passage attenuated WC11 strain. **First**, the full sequence of the AlHV-1 BAC clone was obtained using 454 deep sequencing. As expected, the obtained sequence for the AlHV-1 C500 BAC strain was nearly identical to the sequence of the C500 reference strain. However, we identified the translocation of a duplicated region containing ORF50, A6 gene and part of A7 gene flanked by prDNA units. Restriction profile, southern blotting and PCR analyses showed that the duplicated region was separated from the left end of the LUR by one unit of prDNA. We used the BAC clone to remove the translocated region in order to determine whether the duplication of ORF50 and A6 could increase viral fitness *in vitro* and/or *in vivo*. **Secondly**, the full sequence of the WC11 strain was obtained using an Illumina sequencing approach. Although the genome of the WC11 strain was nearly identical to the reference C500 sequence, two regions were deleted. First, a 1,175 bp sequence consists in the left end of the LUR including the predicted A1 gene. Second, a 2,174 bp sequence includes the full coding sequence of A7 gene and the first part of A8 gene. A high sequence divergence was also observed in the right-end of the LUR, a region including the A9.5 and A10 coding sequences. Interestingly, a translocation of a duplicated region containing ORF50 and A6 was also present in the WC11 genome. The genes modified or absent in the WC11 strain are particularly susceptible to be involved in the induction of the disease. While the duplication of ORF50 could have been selected *in vitro* to enhance viral growth, the potential function of the A1, A9.5, and A10 genes are unknown. However, A7 and A8 genes are positional homolog to the Epstein-Barr virus genes encoding gp42 and gp350. As such, A7 and A8 are potentially involved in the entry of the virus and in the regulation of the viral tropism. Viruses impaired for the expression of A7 and/or A8 are currently in production in order to study their functions in MCF induction.

41.

Caps-It: An automated platform for multi-parameter data collection on live pathogens of higher or unknown biosafety risk.

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Research work on or with live pathogens of higher or unknown biosafety risk requires elaborate measures to protect both the operator as well as the environment. Taking the operator out of the space in which the pathogens are being handled, allows to condense the required facility volume (and thus footprint) to an absolute minimum. This reduction in turn results in an even larger reduction in the surface of the containment barrier, and thus overall investment.
In a biosafety environment, there are only two feasible automation approaches. The first one is to avoid automation at all cost to reduce mechanical errors to a minimum. However, in this context, the error rate, flexibility and throughput are completely in the hands of humans, and one must keep in mind that all instruments require service or repair in due time. In the second approach, the automation level is maximized to such an extent that mechanical errors do not affect the overall functionality of the system any more. Furthermore, advanced automation allows the system to carry out multiple studies at the same time, and to maximize the use of the available instrument capacity by 24/7 operation, in most instances without requiring direct supervision by an operator. Intermediate levels of automation will only suffer from the disadvantages of both.
The Caps-It project is a fully automated, generic instrument configuration that allows microtiter plates to circulate between incubators (with the appropriate atmospheric conditions for the pathogens to grow), liquid handlers (which allow to add substances and harvest samples), plate washers (for removal or replacement of liquids), plate readers (to collect whole-well absorbance, luminescence or fluorescence data) and confocal high-content imagers (to acquire microscopic images at the cell or subcellular level). The robotics system is enclosed by an isolator system with the appropriate air, liquids and solids handling to contain, at all times, the pathogens that are being handled.
The project was approved by the Hercules Foundation in 2014 and, at present, the construction of the robotics system has been completed, the construction of the isolator system has been started, and the construction works on the facility are currently ongoing. The isolator system will be set up in the new BLS-3 facility of the Rega Institute for Medical Research, will be connected to a BLS-3+ lab cluster and will be validated for decontamination with chlorine dioxide.

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42.

Assessment of the immunosuppressive effect of H9N2 virus infection in SPF chickens

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Abstract
H9N2 Avian influenza virus (AIV), a major cause of economic losses, is massively circulating in the poultry population of the whole Asia and it affects poultry also in Northern Asia, and, more sporadically in Europe. In this study, pathogenicity, tissue dissemination of H9N2 A/chicken/Israel/1163/2011 in various organs, shedding and the early immune response in H9N2 inoculated specific pathogen free (SPF) chickens were examined to explain the virus pathogenesis. Furthermore, to investigate whether the infection with H9N2 virus might have immunosuppressive effect or not, a model to assess immunosuppressive effect of H9N2 infection in chickens was developed using splenocytes and peripheral blood mononuclear cells and different mitogens.
Infected chickens exhibited mild clinical signs, the hemorrhage in liver and swelling spleens were observed. Viral RNA was detected in swabs, lungs, kidneys, duodenums and spleens. At 10 dpi, all chickens were seroconverted. Ex vivo stimulation experiments with different mitogens were done. Then ChIFNg production evaluated by Elisa was used to measure the H9N2 specific cell-mediated immunity. The results reveal that at 4 and 5 dpi, there was only weak proliferative response in the infected chickens while high proliferation of mitogen-activated splenocytes and PBMC was observed in the negative control chickens. Incidentally the initial H9N2 virus stock was found to be contaminated with Newcastle disease virus (NDV) with the proven dose of $1.35 \times 10^{0.5}$/infection dose for each chicken. Six animals out of all experimentally infected chickens showed excretion via cloacal route at different days post infection. However, no seroconversion was detected on the experimentally infected chickens. In conclusion, the clinical and virological data from this study showed the mild virulence of the H9N2 A/chicken/Israel/1163/2011 virus for SPF chickens and the multi-organic tropism of the virus. Immunosuppressive ability of virus strain was recorded on splenocytes at 4, 5 dpi and PBMC at 5 dpi. This result will be confirmed with complementary analyses in vitro, infecting splenocytes and PBMC cultures with the H9N2 virus and monitoring the immune response. We will conduct a relevant experiment using purified H9N2 by plaque assay to evaluate the effect of NDV presence on H9N2 virulence and shedding.

43.

Epigenetic regulation of the viral RNA telomerase subunit over-expressed in lymphoma induced by Marek's Disease Virus

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Marek's disease virus serotype-1 (MDV-1) is an oncogenic chicken alphaherpesvirus that induces the rapid onset of a highly malignant CD4+ T-lymphoma, consistent with the involvement of one or more virus-encoded oncogenes. Disease pathogenesis begins with a semi-productive infection, followed by a switch to latent infection. The transformation of latently infected CD4+ lymphocytes is the ultimate consequence of interactions of MDV with the host cells. This study focuses on the mechanisms involved in this switch between productive and latent phase of MDV life cycle. The objectives are to determine whether epigenetic modifications are involved in this transition and to establish patterns of DNA methylation in the promoters of lytic and latent genes involved in the regulation of MDV life cycle. Methylation patterns were established for three genes of interest, 38 kDa phosphoprotein (pp38), 14 kDa phosphoprotein (14 kDa) and viral telomerase RNA subunit (vTR) at the key steps of the viral infection. The impact of DNA methylation was assessed through promoter reporter assay. Bisulfite Genomic Sequencing Assay (BGSA) was used to assess pp38, 14 kDa and vTR promoter methylation patterns at three relevant steps of MDV life cycle in vitro. Reporter vectors were constructed to evaluate the activity of vTR, pp38 and 14 kDa promoters. Luciferase activity of non-methylated promoter versus methylated ones was measured. Finally, response elements to specific transcription factors were studied in the vTR promoter through mutagenesis of c-Myc binding sites. A correlation was noticed between the level of methylation observed in the studied promoters and the expression level of the corresponding genes. The pp38, 14kDa and vTR promoters analyzed by BGSA showed high levels of methylation of CpG dinucleotides during latency and it was shown that DNA methylation induces transcriptional silencing of lytic genes while demethylation is associated with reactivation of the gene expression. DNA methylation positively influences the expression levels of vTR and the activity of the viral induced telomerase. The results showed that demethylation is associated with decreasing telomerase activity. The transcription of genes associated with the replication (pp38) was significantly induced after DNA demethylation. Using the same demethylation agent on vTR its activity was considerably decreased. Impact of DNA methylation was then analyzed by luciferase promoter reporter assay. There was significantly lower activity of methylated promoters compared to non-methylated ones. Site directed mutagenesis demonstrated that the cellular transcription factor c-Myc plays a role in vTR promoter activity. The effects of mutations were masked by methylation. The study of genes of interest is ongoing in samples collected in vivo. Methylation patterns will be established in genes associated with latent phase (vTR, LAT) and genes associated with productive phase (ICP4, pp38) from different cell types such as feather follicle epithelial cells, peripheral blood leukocytes and magnetically sorted CD4+ and CD30+cells.

44.

Persistent shedding of murine norovirus in stool is reduced to undetectable levels by the polymerase inhibitor 2'-*C*-methylcytidine without emergence of drug-resistant variants

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Human noroviruses are the leading cause of foodborne illness and are responsible for >50% of gastroenteritis outbreaks worldwide. There are no vaccines or antiviral drugs available. Potent and safe antiviral therapy is urgently needed to reduce the burden of norovirus disease in vulnerable populations – young children, the elderly and immunocompromised – where prolonged and severe disease occurs and norovirus gastroenteritis may become chronic, linked to morbidity and mortality. Potent antivirals may also have an important place as prophylaxis i.e. to reduce transmission and to prevent infection during an outbreak.
We previously reported that the viral polymerase inhibitor 2'-*C*-methylcytidine (2CMC) and the broad-spectrum anti-RNA virus drug favipiravir (T-705) inhibit the *in vitro* replication of norovirus (Rocha-Pereira et al. 2012 BBRC 427(4): 796-800; BBRC 424(4): 777-780.) Using IFN receptors deficient- AG129 mice, we demonstrated that 2CMC efficiently protects against MNV (MNV-1.CW3) -induced diarrhea and mortality (Rocha-Pereira et al., 2013, J Virol 87:11798-805).
We here established a mouse model for persistent norovirus infection using the murine norovirus (MNV/CR6 strain). IFN receptors deficient- AG129 mice were infected orally with the CR6 strain; this asymptomatic infection results in shedding in the stool of high titres of CR6, starting 6 days post infection and lasting weeks to months. Starting at 7 days post infection, the inhibitory effect of 2CMC (100mg/kg/day sc) and T-705 (200mg/kg/day, oral gavage) on viral shedding in stool was assessed. 2CMC was able to reduce the shedding of virus in the stool to undetectable levels when treatment lasted 7 to 14 days. However, 3 days after stop of 2CMC-treatment, viral RNA became again detectable in the stool of mice, at levels comparable to those of the untreated control. Next, the effect of a treatment scheme consisting of two rounds of 14 consecutive days of either 2CMC- or T-705-treatment (with a pause of 4 weeks in between) was evaluated. 2CMC, but not T-705, reduced the MNV-shedding in the stool to undetectable levels after the 14-day treatment. The second round of 14-day treatment with 2CMC proved again effective, the virus susceptible to the antiviral, suggesting that no drug-resistant variants had emerged. Favipiravir, despite inhibiting *in vitro* MNV replication, was again not effective in the mouse model.
The activity of 2CMC in this chronic mouse infection model suggests that a (more) potent inhibitor of norovirus replication would be able to at least reduce viral replication in patients with chronic norovirus infections. In such patients, antiviral drugs with a high barrier to resistance (like 2CMC) should be used.
In conclusion, we demonstrated for the first time that an inhibitor of norovirus replication is able to reduce viral shedding in chronically infected animals to undetectable levels. This validates this model for the evaluation of the potential *in vivo* efficacy of novel norovirus inhibitors.

45.

In vivo characterization of the RNAPIII-dependent BLV miRNAs cluster

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Bovine leukemia virus (BLV), a B-lymphotropic oncogenic retrovirus, is the etiologic agent of enzootic bovine leucosis. The prevalence of BLV infection remains high outside Western Europe where it causes drastic economical losses. In addition, BLV shares common biological and structural features with the human T-cell leukemia viruses I and II (HTLV-I and II), thereby representing a good model to study tumor formation induces by retroviruses.

It is widely accepted that BLV latency, due to the RNA polymerase II 5'-LTR-driven transcriptional and epigenetic repression is a viral strategy to escape from the host immune system and allow tumor development. Recently, it has been demonstrated by deep sequencing and bioinformatics analyses that the BLV genome encodes a cluster of micro-RNAs (miRNAs) which is predicted to be transcribed by RNA polymerase III (RNAPIII) [1, 2], suggesting that the silencing dogma in BLV transcriptional regulation is only partially correct.

Here, we demonstrated, by chromatin immunoprecipitation (ChIP) assays, the *in vivo* recruitment of RNAPIII to the BLV miRNAs cluster both in BLV-infected cell lines and in ovine BLV-infected primary cells. We next demonstrated, for the first time, a functional link between RNAPIII transcriptional initiation and viral miRNAs expression, using RNA interference experiments. In addition, we showed that RNAPIII is recruited through a canonical type 2 RNAPIII promoter similar to the one directing tRNAs transcription. We also showed that both tumor- and quiescent-related isoforms of RNAPIII RPC7 subunit were recruited to the BLV miRNAs cluster whereas interestingly, in BLV-infected ovine primary cells, only the tumor-related isoform was recruited to this RNAPIII promoter. Finally, our epigenetic ChIP studies demonstrated that the BLV miRNAs cluster was enriched in positive epigenetic marks such as DNA hypomethylation and histone acetylation, which is in agreement with the high miRNAs expression level previously reported in BLV-infected cells.

Overall, our results provide new insights into a better understanding of the molecular mechanisms regulating gene expression of oncogenic retroviruses.

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46.

A herpesvirus alters the behavior of its host to enhance its replication

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When infected by pathogens, endotherms and ectotherms can both increase their body temperature to limit the infection. Ectotherms do so by moving to warmer places, hence the term “behavioral fever”. Previous studies of the regulation of behavioral fever in ectotherms at the level of the central nervous system have demonstrated its evolutionary relationship with fever in endotherms. However, the function of cytokines as peripheral mediators of this process is still unknown. It is also unknown whether pathogens can alter this behavior to increase their fitness. Here, we show that common carp express salutary behavioral fever in response to cyprinid herpesvirus 3 (CyHV-3) infection and that Tnfα1 is a key mediator of this innate mechanism in the CyHV-3/carp model. Our results demonstrate that fever in endotherms and behavioral fever in ectotherms are evolutionarily and functionally related through cytokine mediators that originated more than 400 million years ago. We also demonstrate that CyHV-3 is able to delay the migration of infected fish to warmer environments and thereby promotes its replication. The mechanism of this inhibition was found to rely on the expression of ORF12 encoding a viral soluble Tnfα-receptor. Surprisingly, ORF12 deletion did not exhibit a phenotype under standard laboratory conditions but only revealed its function under conditions mimicking temperature gradients of natural environments. Remarkably, this study demonstrates for the first time the ability of a vertebrate virus to alter host behavior through the expression of a single gene.

47.

New antivirals against hRSV

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Human respiratory syncytial virus (hRSV) is the most important cause of acute airway infections in infants and young children. By the age of two nearly all children will have undergone at least one hRSV infection. Although usually causing only mild disease, in a fraction of patients (1-2%) hRSV infection leads to serious bronchiolitis where hospitalization is required. It has been estimated that each year 160.000 children die due to hRSV infection. No effective prophylactic vaccine and no hRSV-specific therapeutic small molecule is clinically developed. The only way in which high-risk infants can be partially protected from a severe hRSV infection is by monthly injections with a humanized mouse monoclonal antibody directed against the pre- and postfusion conformation of the Fusion protein (F) of hRSV. It was shown that conventional antibodies specifically binding to the prefusion conformation of F are much more potent hRSV neutralizers than antibodies directed against the postfusion conformation. Our aim was to isolate single domain antibodies directed against epitopes in F that are unique to the prefusion conformation. Following immunization of a llama with recombinant prefusion-stabilized F and a screening strategy based on bio-panning and semi-high-throughput virus neutralization, we isolated and characterized several F-specific recombinant single domain antibodies. Two of these single domain antibodies neutralized hRSV A and B strains with half maximum effector concentrations in the low picomolar range. Crystallization data show that these two candidates, although differing in CDR3 sequence, bind the same pocket on the F protein. In *vivo* studies proved that these molecules effectively protect mice from RSV challenge when delivered prophylactically, experiments to test the therapeutic potential of these novel candidate antivirals are ongoing.

48.

Novel enterovirus infection model for the *in vivo* study of antiviral therapy and the development of resistance.

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Although many enteroviruses (*Picornaviridae* family) are important human pathogens (such as poliovirus, enterovirus 71 and rhinoviruses), no antiviral therapy is currently approved for the treatment of entero- or rhinovirus infections. Here we report the development of a robust and relevant enterovirus infection model that can serve (i) to evaluate the antiviral effect of newly identified compounds in small animals and (ii) to study the possible development of resistance. SCID mice were intraperitoneally (ip) injected with Coxsackievirus B4 (CVB4). From day 3 post infection (p.i.) on, diarrhea and weight loss were observed. At day 5 p.i. the mice needed to be euthanized. A high viral titer (~10^9 GE/100 mg tissue) was detected in the pancreas, whereas in other organs like heart, lung, liver, spleen and brain relatively low virus titers were observed (~10^6-10^7 GE/100 mg tissue). High levels of viral RNA (10^9 GE/100 mg tissue) and infectious particles (10^6 $CCID_{50}$/ml) were detected in the serum as well. Next, we aimed to explore whether this model can be used to evaluate the effect of long-term antiviral treatment and the development of drug resistance. We therefore studied the antiviral activity of compound A (a proprietary 2C-targeting compound discovered in our laboratory). CVB4-infected mice were treated according to three different regimens: (group A) 20 mg/kg/day during 15 consecutive days starting at the day of infection, (group B) 20 mg/kg/day during 20 consecutive days starting at the day of infection and (group C) 20 mg/kg/day during 20 consecutive days starting at day 1 p.i. All mice remained healthy throughout the entire course of the treatment and at least an additional 18 days after cessation of treatment. At the end of the experiment (day 77 p.i.), the survival rate of group A and B was 60% whereas that of group C was 0%. To assess whether the (delayed) lethality was due to the emergence of escape-mutants, we isolated viral RNA from the serum, heart and pancreas of animals belonging to all groups after which we sequenced the 2C gene. Interestingly, all evaluated test samples showed the presence of one substitution A239T/V in the 2C protein. This mutation did not result in a reduced sensitivity to the compound in *in vitro* antiviral assays. Next generation sequencing revealed the presence of an additional substitution in the 3D-polymerase gene (T48P) in the virus isolated from the mice of only group C. The possible role of these mutations in the re-emergence of lethality still need to be investigated. In conclusion, we developed a robust, lethal CVB4 infection model that can be used to study CVB4 population dynamics over time in the infected host and could reveal how minority variants within a population can be expanded under (sub)optimal antiviral pressure.

49.

Antibody dependent macrophage mediated phagocytosis and trogocytosis of influenza and HRSV infected cells, visualized by live cell imaging.

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Influenza and the Human Respiratory Syncytial Virus (HRSV) are two major human respiratory pathogens. Current influenza vaccines aim at neutralizing virus by the induction of antibodies that target the globular head of the hemagglutinin (HA) protein. However because HA is very prone to genetic drift influenza can rapidly escape vaccine induced neutralizing antibodies. Despite decades of research, there is currently no vaccine for HRSV. A major obstacle in the development of an effective HRSV vaccine is the ability of this virus to infect humans in the presence of neutralizing antibodies.

In our lab we have developed an alternative vaccination strategy based on non-neutralizing antibodies directed against small but highly conserved viral antigens that are expressed at the surface of infected cells. In contrast to HA and NA the ectodomain of the small influenza M2 protein (M2e) is not subjected to antigenic drift. Hence anti-M2e antibodies can protect against a broad range of influenza strains. Recently, we have developed a new HRSV vaccine candidate that induces protective antibodies directed against the ectodomain of the Small Hydrophobic protein (SHe).

Although M2e-and SHe-specific antibodies can respectively protect against influenza and HRSV infections, these antibodies do not neutralize viruses in vitro. In contrast, both M2e- and SHe-specific antibodies strongly depend on FcγReceptors and alveolar macrophages to protect against infection. Therefore we hypothesize that M2e- and SHe-specific antibodies control viral infections by engaging alveolar macrophages to eliminate virus infected cells. Using live cell imaging, flow cytometry and confocal microscopy we could demonstrate that in the presence of M2e- or SHe-specific antibodies macrophages can indeed phagocyte infected cells in vitro. This activity was significantly reduced in alveolar macrophages derived from FcγRI/III$^{-/-}$ mice. In addition, we could demonstrate that vaccination of humans with an universal M2 vaccine enables antibody-dependent macrophage mediated phagocytosis of M2 expressing cells. These data support the hypothesis that antibodies directed against small viral antigens that are expressed at the surface of infected cells can control pulmonary virus infection by eliminating infected cells through macrophage mediated phagocytosis.

Surprisingly, time lapse imaging revealed that , next to phagocytosis of infected cells, M2e antibodies can also engage macrophages to take up M2 antigen directly from the surface of target cells via a process that resembles trogocytosis. Via this process macrophages are able to remove a significant portion (70-90 %) of M2 antigen from the cell surface. We are currently investigating the cellular details this process by correlative Light and electron microscopy.

50.

Self-inhibition of synthesis reduces antigen presentation of the alcelaphine herpesvirus 1-encoded latency-associated protein, aLANA

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Alcelalphine herpesvirus 1 (AlHV-1) persistently infects its natural host, the wildebeest, without inducing any clinical signs. However, cross-transmission to other ruminant species leads to the development of a deadly lymphoproliferative disease named malignant catarrhal fever (MCF). AlHV-1 ORF73 encodes the latency-associated nuclear antigen (LANA)-homolog protein termed aLANA. Similarly to other viral genome maintenance proteins encoded by gammaherpesviruses, aLANA has recently been shown to be essential for viral persistence and induction of MCF. Here we have investigated the self-inhibition of antigen presentation by aLANA and the potential role of such mechanism during the development of MCF. We showed that the GE-rich repeat domain of aLANA was sufficient to inhibit the presentation of an epitope linked to it. Though antigen presentation in absence of GE was dependent upon proteasomal degradation of aLANA, a lack of GE did not affect protein turnover. We further found that similarly to EBNA-1 GAr, aLANA GE downregulated protein self-synthesis. Likewise, such mechanism could be associated with reduced antigen presentation *in vitro*. In addition, in-frame insertion of GE repeat domain in a heterologous eGFP protein significantly down-regulated protein steady-state levels and self-antigen presentation. Next, we modified the AlHV-1 ORF73 gene sequence to reduce the purine bias in GE, without affecting the peptidic sequence. Such codon-modified aLANA GEm construct displayed increased antigen presentation. Finally, we generated an AlHV-1 recombinant strain expressing a GE-deficient aLANA protein and observed that viral growth was not affected *in vitro* by the absence of aLANA GE domain and MCF could be induced in rabbits irrespective of the expression of full-length aLANA or GE-deficient aLANA protein.

51.

Antiviral activity of broad-spectrum and enterovirus-specific inhibitors against clinical isolates of enterovirus D68

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Human enterovirus D68 (EV-D68), first identified in 1962 in the United States, is a single-stranded, positive-sense RNA virus that belongs to the enterovirus genus in the family *Picornaviridae*. Starting from 2008, EV-D68 outbreaks were observed more frequently worldwide. In 2014, EV-D68 caused the largest outbreak until now in the United States, with >1,100 people diagnosed with EV-D68 induced respiratory illness. Those affected were mostly children with asthma or a history of wheezing. Occasionally, the virus has also been associated with polio-like illness, such as acute flaccid paralysis (AFP) and limb weakness. So far, no vaccines or antivirals are available for the prophylaxis or treatment of EV-D68 infection. Hence, effective and safe drugs against EV-D68 infection are sought.

We carried out a comparative study of the *in vitro* antiviral activity of known enterovirus-specific and broad-spectrum inhibitors against a selection of EV-D68 strains. To this end, a panel of 10 clinical isolates from The Netherlands, Thailand and the United States (from the 2014 outbreak) was selected consisting of representative strains of the three major genogroups. The antiviral activity of compounds against this panel of EV-D68 strains was assessed in a cell-based cytopathic effect (CPE) reduction assay with the MTS/PMS method. The 3C-protease inhibitor rupintrivir proved to be an efficient inhibitor of the *in vitro* replication of all EV-D68 isolates, with mean 50% effective concentration (EC_{50}) ranging from 0.0018 to 0.0030 μM. Likewise, the protease inhibitor SG85 also efficiently inhibited all EV-D68 strains with EC_{50}s ranging from 0.0022 to 0.0080 μM. Enviroxime, which targets the cellular phosphatidylinositol 4-kinase IIIβ, inhibited the replication of all tested EV-D68 isolates, with EC_{50}s between 0.19 and 0.45 μM. The capsid binder pleconaril was active against all EV-D68 strains from the reference panel (with EC_{50}s between 2.4 and 9.6 μM), including EV-D68 strains that circulated in the United States in 2014. Also vapendavir showed antiviral activity against EV-D68, with exception of one of the cluster A isolates that proved to be resistant to the antiviral effect of vapendavir. Interestingly, an analogue of vapendavir, pirodavir, was not able to completely inhibit the replication of the EV-D68 isolates tested. Favipiravir, a drug that has been approved in Japan for the treatment of infections with influenza virus but that also exerts activity against other RNA viruses, including Ebola virus, proved to be a weak inhibitor of the *in vitro* replication of EV-D68 ($EC_{50} \geq 63$ μM). The data presented here offer a framework of reference data against which other inhibitors of EV-D68 replication can be compared.

52.

POST-EXPOSURE TREATMENT WITH ANTI-RABIES VHH AND VACCINE TO PROTECT MICE FROM RABIES

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Rabies virus is a model neurotropic RNA virus causing an aggressive and lethal infection in mammals. In humans, disease can effectively be prevented by pre- or post-exposure prophylaxis. Pre-exposure prophylaxis consists of vaccination only, whereas post-exposure prophylaxis consists of a combination of vaccination and treatment with anti-rabies immunoglobulins. The latter are very expensive, scarce in supply and often unaffordable for people living in endemic areas. Current anti-rabies immunoglobulin preparations are still purified from the plasma of vaccinated persons. The World Health Organisation therefore strongly recommends the development of alternatives.

VHH (or Nanobodies®) are the smallest functional fragments of heavy chain antibodies naturally occurring in Camelids. They are very stable and can be fused into multimeric constructs[1,2,4]. Previously, we developed anti-rabies VHH with high affinity for the viral surface glycoprotein using selection by phage display. We showed that anti-rabies VHH were able to neutralize the virus *in vitro* and *in vivo*, and half-life extended anti-rabies VHH could protect mice from infection when administered soon after virus inoculation.

Here, we examined the added value of combined treatment of anti-rabies VHH with the rabies vaccine for post-exposure prophylaxis against rabies in mice. Mice were first infected using intranasal inoculation of a lethal dose of rabies virus, and received the vaccine on day 1 and 3 intramuscularly as is common in humans. Mice were also treated with anti-rabies VHH (1.5 mg, IP) on day 1. Combined treatment was able to rescue 60 % of the animals from lethal challenge, in contrast to a survival rate of 0 % and 19 % when mice were treated respectively with vaccine or anti-rabies VHH alone. Prophylactic vaccination 4 weeks before the infection rescued 50% of the mice. Thus the combined treatment with anti-rabies VHH and vaccine directly after the viral challenge seemed more successful than preventive vaccination. This emphasizes the importance of rapid neutralisation of the virus by passively administered VHH soon after challenge.

In conclusion, post exposure treatment with anti-rabies VHH and vaccine proved superior to single treatment with either anti-rabies VHH or vaccine alone, and we believe that anti-rabies VHH can be a valuable alternative for human rabies immunoglobulins. Since VHH are easier to produce on an industrial scale and have better thermal stability than conventional antibodies, they offer important advantages over the currently used anti-rabies immunoglobulin preparations.

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53.

Successive subclinical group A and C rotavirus infections in piglets after weaning on a closed Belgian pig farm

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Diarrhea is an important health problem in young pigs and can be caused by rotaviruses. Within genus *rotavirus*, 8 different species (A to H) are officially recognized. Until now RVA is considered clinically and epidemiologically most important. In the past decades, the presence and impact of rotaviruses in Belgian swine herds has not been investigated.
In the present pilot study, 9 weaned piglets of a closed Belgian pig farm (1000 sows) were followed for their excretion of RVA, RVC and *Escherichia coli* during the first 24 days postweaning. The farm had a history of bloody diarrhea in the weaning barn and zinc oxide and colistine feed medication, and acidification of drinking water were used prophylactically. At 2 day time-intervals, the farm was visited by the first author, a fecal score was given and weight was determined. Rectal swabs were collected to follow-up quantitatively RVA and RVC excretion using RT-qPCR. Strains shed were characterized for the genes encoding outer capsid proteins VP7 and VP4, important for the induction of neutralizing antibodies. *E. coli* was isolated under aerobic conditions.
No explosive diarrhea was seen during the entire study period. Interestingly, all piglets became infected with RVA and RVC shortly after weaning. Piglets farrowed by gilts shed RVA/RVC at the day of weaning, but at low viral loads. At least two successive RVA replication waves were noticed in all pigs, but the area under the curve of the second wave was lower than that of the first wave. This might be an indication for rapid induction of cross-protection. Strains of the two replication waves were genetically distinct and possessed the G5P[13] and G9P[23] genotype combination for VP7 and VP4. During the first 10 days after weaning, RVC excretion was less pronounced than RVA. In contrast, RVC was shed at higher loads at the end of the study period, when RVA excretion was generally lower. At least two or three different RVC strains (G6P5, G1Px...) were found. Hemolytic *E. coli* were only isolated at scattered time points, whereas non-hemolytic *E. coli* was found at almost all time points. Growth was hampered on this farm, especially in the first week after weaning. In some of the pigs shedding the highest load of RVA, the poorest growth was observed.
In this study, it was demonstrated that weaned piglets can be successively, but subclinically infected with genetically distinct RVA and RVC strains. It seems that rapid cross-protection is being induced between genetically distinct RVA strains. Despite the presence of rotavirus on this farm, there was no explosive diarrhea observed. Most likely, the prophylactic treatments suppressed the proliferation of pathogenic enteric bacteria, and prevented a severe outbreak of diarrheic problems. The growth retardation might be a complex problem in which rotaviruses play a role. Nonetheless, the pathotypes (diarrhea, growth retardation or asymptomatic) of different rotavirus strains will be investigated during ongoing field studies, so that accurate preventive measures can be developed.

54.

Mapping the lncRNAome during the HIV replication cycle suggests a role in proteosomal and apoptotic pathways

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Abstract
Studying the effects of HIV infection on the host transcriptome has typically focused on protein-coding genes. However, recent advances in the field of RNA sequencing revealed that long non-coding RNAs (lncRNAs) add an extensive additional layer to the cell's molecular network and are highly involved in the regulation of molecular processes, including gene expression. Here, we performed transcriptome profiling throughout a primary HIV infection *in vitro* to investigate lncRNA expression at the different HIV replication cycle processes (reverse transcription, integration and particle production). Subsequently, guilt-by-association, transcription factor and co-expression analysis were performed to infer biological roles for the lncRNAs identified in the HIV-host interplay.
Many lncRNAs were shown to play a role in mechanisms relying on proteasomal and ubiquitinylation pathways, apoptosis, DNA damage responses and cell cycle regulation. Through transcription factor binding analysis, we found that lncRNAs display a distinct transcriptional regulation profile as compared to protein coding mRNAs, suggesting that mRNAs and lncRNAs are independently modulated from each other. In addition, we identified five HIV linked co-expression events with possible *cis* regulatory lncRNAs that control the nearby mRNA expression and function.
Altogether, the present study demonstrates that lncRNAs add a new dimension to the HIV-host interplay and should be further investigated as they may represent targets for controlling HIV replication.

55.

Influenza A virus escape routes from immune selection by monoclonal antibodies directed against the ectodomain of matrix protein 2

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The conserved ectodomain of matrix protein 2 (M2e) of influenza A viruses is a universal influenza A vaccine candidate. However, natural M2e-specific immunity is very weak. Therefore, it is interesting and important to explore the potential evasion strategies of influenza A viruses under M2e-based immune selection pressure that is induced by vaccination.
We treated Severe Combined Immunodeficient (SCID) mice with anti-M2e mouse IgG monoclonal antibodies and challenged them with PR8 virus. Mice were treated with mAb65 (IgG2a), mAb37 (IgG1), mAb148 (IgG1) or isotype control monoclonal antibodies. mAb65 and mAb37 recognize the same internal epitope in M2e and bind to M2e with the same affinity. In contrast, mAb148 binds to the highly conserved N-terminus of M2e. Viral load in the bronchoalveolar lavage (BAL) fluid of these mice was determined by plaque assay. In addition, we determined the viral genetic diversity by Illumina MiSeq next-generation sequencing (NGS).
SCID mice infected with PR8 virus and treated with M2e-specific mAb65, mAb37 or mAb148 survived significantly longer than isotype control mAb-treated mice. M2e-specific IgG2a protected significantly better than IgG1 and even resulted in virus-clearance in some of the SCID mice. NGS-analysis of the virus population that persisted in mAb37- or mAb65-treated mice revealed that viruses emerged with a mutation at positions proline 10 or isoleucine 11 in M2. These mutations were Pro10His/Leu and Ile11Thr, abolishing recognition by mAb37 and mAb65, and occurred at diverse frequencies, either alone or combined in the viral population. Remarkably, in half of the BAL samples isolated from moribund mAb37-treated mice and in all mAb148-treated mice, virus was isolated with a wild type M2 sequence but with non-synonymous mutations in other viral proteins, mainly the polymerases and/or the hemagglutinin. These data suggest that compensatory mutations represent an alternative escape route for the virus from M2e-mediated humoral immunity in SCID mice. We have initiated a reverse genetics approach to investigate how these mutations contribute to escape from M2e-specific monoclonal antibody therapy.

56.

A retrospective study on equine herpesvirus type-1 associated myeloencephalopathy in France (2008–2011)

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Abstract
Diagnosis of equine herpesvirus-1 associated myeloencephalopathy (EHM) can be troublesome, but early recognition and knowledge of risk factors are essential for prevention and control. The objectives for this study are to (1) describe EHM in France, (2) improve clinical recognition, (3) identify risk factors. Through epidemiosurveillance of acute neurological cases (all considered to be potentially infectious cases) in France (2008–2011), 26 EHM cases were identified and 29 EHM negative control cases. EHM cases were described and compared to controls with univariate, multivariate and classification and regression tree analysis. EHM cases had a 46% fatality rate and were frequently isolated cases. Most showed ataxia, paresis and a cauda equina syndrome, yet presence of other neurological signs was variable. Statistical analysis identified the following variables to be significantly associated to EHM compared to controls: introduction of a new horse to the herd, cauda equina syndrome, larger herd size, saddle horses and month of occurrence. The presence of many isolated cases, and less typical and variable clinical presentations emphasize the difficulty in diagnosing EHM. Nevertheless, history and clinical examination of acute neurological cases can be valuable in recognizing EHM early as well in order to select those cases that need further laboratory testing and infection control measures. Moreover, with a different study format and geographic location, risk factors were found to be similar to previous studies, therefore strengthening their significance to the spread of EHM.

57.

Proteomic and functional analysis of Cyprinid herpesvirus 3 envelope transmembrane proteome

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Cyprinid herpesvirus 3 (CyHV-3) is the aetiological agent of a lethal disease in koi and common carp. Herpesvirus envelope transmembrane proteins play essential roles in many processes such as entry, immune evasion mechanisms, morphogenesis and egress of progeny virions from the infected cells. Moreover, due to their exposure on the virion surface, they represent potential targets for neutralizing antibodies. The initial goal of the present study was to identify the transmembrane envelope proteins of CyHV-3 and to determine those that are essential for viral growth *in vitro*. With that goal in mind, we investigated the protein composition of CyHV-3 extracellular virions using 2D LC-MS/MS mass spectrometry approaches, and then addressed the effect on viral growth *in vitro* of individual deletion of the open reading frame (ORF) encoding predicted transmembrane envelope proteins. This approach led to the identification of 16 transmembrane proteins, among which 8 turned out to be essential for viral growth *in vitro*. The qualitative effect of deletion of non-essential envelope proteins on viral growth was investigated by multistep growth assay and plaque assay. Deletion of ORF25 was shown to reduce the production of extracellular virions while deletion of two other ORFs (ORF148 and ORF149) delayed the kinetic of virion production. Plaque size was reduced by deletion of ORF25 and ORF149. Finally, the potential of essential transmembrane proteins as target for neutralization was investigated. Polyclonal antibodies were produced against the 8 essential proteins by DNA immunization of mice. These antibodies allowed the study of the subcellular localization of these proteins and their potential as target for neutralization. Antibodies raised against 4 essential proteins (ORF32, 99, 115 and 131) exhibited neutralising activity.

The present study represents a major breakthrough for the understanding of the biology of the archetype of the *Alloherpesviridae* family and for the development of prophylactic methods against this economically important virus.

58.

LABORATORY VALIDATION OF TWO REAL TIME RT-PCR METHODS FOR FOOT-AND-MOUTH DISEASE VIRUS WITH AN INCREASED DIAGNOSTIC SPECIFICITY

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Introduction

The 3D and 5'-UTR rRT-PCRs from Callahan et al. (2002) and Reid et al. (2002) are commonly used reference methods for the detection of FMDV RNA. Using both rRT-PCRs in parallel yields an optimal diagnostic sensitivity (King et al., 2006). During the implementation and validation of each of the rRT-PCRs in our laboratory we observed excellent results for the analytical sensitivity and specificity, the repeatability and reproducibility and the linearity of the assay. Using reference samples we observed excellent diagnostic sensitivity and specificity. However, when field samples were submitted for differential diagnosis we regularly encountered difficulties to declare these field samples free from FMDV due to linear increases in fluorescence at the end of the amplification phase with the cut-off value for positive set at Cp 38 (Cp 38-45: doubtful test result). Our aim was to improve the diagnostic specificity of both rRT-PCR methods without reducing the cut-off value for positive (Cp 38).

Materials and methods

Both rRT-PCRs were run in parallel in a triplex one-step protocol with beta-actin as an internal control (IC) and synthetic RNA as an external control (EC) (Vandenbussche et al., 2010). To increase the specificity of the assay 5' flaps (*i.e.* short 5'AT rich overhangs non-complementary to the target region) were integrated into the forward and reverse primer sequences for FMDV and into the primer sequences for the synthetic RNA control. Further, each amplification cycle consisted of 10' at 95°C and 30' at 63°C, instead of 60°C in the original laboratory protocol, and 50 amplification cycles were performed instead of the original 45.

Results and discussion

For the assessment of the diagnostic specificity, a panel of negative sheep (N=382), cattle (N=372) and pig (N=372) field sera from Belgium was tested. With the cut-off value for positive set at Cp 40, 1 of the 1126 negative samples yielded a false-positive test result in the 3D/IC/EC rRT-PCR (Cp 39.78). No false-positive test result was observed in the 5'-UTR/IC/EC rRT-PCR and no doubtful test result (Cp 40-50) was observed in neither of the two rRT-PCRs. As in the original protocol, excellent results were obtained for the analytical sensitivity and specificity, the repeatability and reproducibility, the linearity of the assay and the diagnostic sensitivity. In conclusion, the results of the validation procedure suggest that the amended rRT-PCR protocols are a reliable tool to declare field samples free from FMDV.

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59.

Implication of DNA methylation in HIV-1 post-integration latency

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The persistence of Human Immunodeficiency Virus type 1 (HIV-1) in a latent state in patients under combined antiretroviral therapy (cART) seriously challenges viral eradication. The transcriptional silencing of the latent HIV-1 provirus is a multifactorial phenomenon including notably a tight epigenetic control[1]. In this regard, the role of DNA methylation in chromatin silencing of HIV-1 promoter, the 5' long terminal repeat (5'LTR), remains controversial. Indeed, two CpG islands flank the HIV-1 transcription start site and have been reported to be hypermethylated *in vitro*, in latently-infected model T-cell lines[2,3]. However, the DNA methylation profile of the HIV-1 promoter is still controversial *in vivo* in patient cells[4-6]. Here, we further investigated the molecular mechanisms underlying the DNA methylation status at the HIV-1 promoter in an *in vitro* HIV-1 latently-infected T-cell line model.

We studied the reactivation potential of several DNA methylation inhibitors, both nucleosidic and non-nucleosidic, in different clones of the HIV-1 latently-infected J-Lat T-cell line model and showed that treatment with cytidine analogs caused the highest reactivations of HIV-1 expression and production. The reactivation levels following demethylating treatment differed in the different J-Lat clones, thereby suggesting that the cellular context of HIV-1 integration influences the reactivation potential of the epigenetic drugs. We next characterized the profiles of demethylation following cytidine analog treatments at the 5'LTR in the different J-Lat cell clones and showed that the CpG dinucleotides were either uniformly demethylated or presented precise and significant hotspots of demethylation. *In silico* analyses at these demethylation hotspots revealed the presence of binding sites for transcription factors. We are currently investigating the specific molecular mechanisms implicated at these hotspots in order to identify new determinants of HIV-1 post-integration latency.

Our results highlight that the demethylation profile at the HIV-1 promoter and the associated reactivation in HIV-1 gene expression and production vary between different *in vitro* latently-infected T-cell lines. These data suggest that different molecular mechanisms could be responsible for the establishment of the HIV-1 promoter methylation pattern, thus providing a potential explanation for the controversy regarding the implication of DNA methylation in HIV-1 post-integration latency *in vivo*.

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60.

Computational analysis of mumps surface proteins reveals their interaction mechanism

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Background

Mumps is a contagious childhood disease caused by the mumps virus (*Paramyxoviridae*). The virus is transferred *via* droplets and the disease is characterized by swelling of the parotid gland. Two major surface proteins, the haemagglutinin-neuraminidase (HN) and the fusion (F) protein, play a key role in the infectious cycle of the virus. Even though little is known about the interaction between the two proteins, several studies suggest that a physical interaction between both proteins is necessary for fusion promotion. Recently, Welch *et al*.[1] identified an electronegative ring in the stalk domain of HN in Parainfluenza virus 5 (PIV5) (*Paramyxoviridae*), which interacts with a positively charged region on the F protein. Additionally, Bose *et al*.[2] found that domain II of the F protein might be involved in HN-F interaction. We have investigated whether this electronegative ring in HN is also present in the mumps virus HN protein, and if the positive region can be found on the F protein of mumps virus and coincides with domain II of the PIV5 F protein.

Methods

We have built homology models of the HN and F protein of the mumps virus with the aid of FoldX. The templates used were 4JF7 and 4GIP, respectively. We have subsequently used PDB2PQR and the APBS tools in Pymol to visualize the electrostatic surface of both proteins.

Results

For both the HN and F protein, we have used homology models to visualize the electrostatic solvent accessible surfaces. The visualization indicates that the region that correlates to the electronegative ring in PIV5 HN is also present in the mumps virus HN protein. Furthermore, in a comparative analysis of this region across 35 members of the *Paramyxoviridae*, we found that the electronegative band is present in this region and is highly conserved. Moreover, through visualization of the electrostatic surface of the F protein, we found an electropositive region in the area that coincides with domain II of the F protein.

Conclusion

Within this research, we have identified an electronegative and an electropositive region in the mumps virus HN and F proteins respectively, as seen previously for PIV5. Moreover, these regions seem to be conserved across the members of the *Paramyxoviridae* which indicates that the proposed mechanism of viral entry of PIV5 applies to all members of the *Paramyxoviridae*.

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61.

Relationship between cytokine expression and viral replication and spread during the early stages of pseudorabies virus infection in pigs.

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Several alphaherpesviruses, including pseudorabies virus (PRV), establish lifelong latency in trigeminal ganglion (TG) neurons of their hosts. The mechanisms involved in latency induction remain however poorly understood. To obtain a better insight in the spread of alphaherpesviruses in their natural hosts and the correlation with the induced immune response shortly after infection, 15 weeks old pigs were intranasally inoculated with PRV strain NIA3. Pigs were euthanized at consecutive days post inoculation and the presence of viral DNA and viral and cytokine related mRNA in different organs is currently being studied using q(RT)-PCR and relative quantification.
Our results indicate a rapid spread of the virus to both peripheral organs (tonsils positive at 1dpi), and the peripheral and central nervous system (CNS). Viral DNA was detected in brainstem, cerebellum and cerebrum at 2, 3 and 5 dpi, respectively. Although the presence of PRV DNA remains to be analyzed in the TG, these results indicate an efficient spread of PRV NIA3 to the CNS without an obvious block at the TG. In the TG, a strong expression of EP0, gB, gC and gE RNA was detected from 3 dpi onwards. By 7 dpi, however, most viral RNA levels were already reduced to undetectable levels. Only low level expression of EP0 RNA remained detectable till 14 dpi (end of study). The rapid decrease of viral mRNA correlated with a substantial increase (>5 fold) of IL-6 and IL-8 mRNA and a moderate increase (3 to 5 fold) of IFN-gamma and TNF-alpha mRNA.
The currently ongoing analysis of expression of other cytokines at the TG and viral and cytokine RNA expression at the periphery and different parts of the CNS during the time course of infection will shed further light on the events taking place during the early stages of PRV infection in its natural host.

62.

HEPARAN SULFATE PROTEOGLYCAN-BINDING CHEMOKINE PEPTIDES AS INHIBITORS OF DENGUE VIRUS SEROTYPE 2, HERPES SIMPLEX VIRUS-1 AND RESPIRATORY SYNCYTIAL VIRUS.

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Many human pathogens including parasites, bacteria and viruses use heparan sulfate proteoglycans (HSPGs) on the surface of target cells for their initial attachment. As such, the interaction between HSPGs and the pathogen may serve as an attractive target for inhibitor development. HSPGs are composed of glycosaminoglycan (GAG) chains covalently attached to a central core protein. HS, a GAG consisting of alternating glucosamine and uronic acid residues, is ubiquitously present on most mammalian cells and mediates the attachment of a number of viruses, including members of the herpesvirus, adenovirus, papillomavirus, retrovirus and flavivirus families. Many polyanions, including heparin/heparan sulfate analogs, have been evaluated for their potential use as antivirals, although the clinical use of these compounds is limited because of anti-coagulant side-effects and poor oral bioavailability. Alternatively, compounds that competitively bind to cell surface HS and mask the virus binding sites may block virus attachment and infection. Chemokines regulate the migration of leukocytes to the site of production and contain a positively charged COOH-terminal region which is believed to bind to GAGs with high affinity to create a chemotactic gradient. The CXC chemokines CXCL9 and CXCL12γ are characterized by a relatively long and basic COOH-terminal region with about 50 % positively charged amino acids. Therefore, we compared the affinity of chemically synthesized COOH-terminal peptides of CXCL9 and CXCL12γ for immobilized heparin using surface plasmon resonance (SPR) analysis and detected binding affinities in the low nanomolar range. Furthermore, we observed that the chemokine peptides exhibited antiviral activity against dengue virus serotype 2 (DENV-2), herpes simplex virus (HSV)-1 and respiratory syncytial virus (RSV) by binding to cellular HSPGs. Finally, we showed that the chemokine peptides compete with domain III of the envelope protein of DENV for binding to heparin. These basic chemokine peptides may be lead molecules for the development of novel antiviral agents.

63.

Accurate quantification of within and between host HBV evolutionary rates requires explicit transmission chain modeling

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To investigate potential differences in hepatitis B virus (HBV) evolutionary rates within and between hosts, Lin *et al*. [1] analyzed the evolutionary dynamics in a known transmission chain. This suggested the HBV evolutionary rate declines with increasing numbers of transmissions, which supports the idea that different evolutionary processes act at the within and between host level [2]. To explain the differential impact on synonymous and non-synonymous substitutions, the authors postulate a `colonization-adaptation-transmission (CAT)' model. However, the estimates that lead to the CAT hypothesis were based on evolutionary rates obtained through pairwise comparisons for various subgroups of the data.

The use of methods that ignore the covariance structure due to shared ancestry prompted us to reanalyze the dataset of [1] in a full probabilistic Bayesian phylogenetic framework (BEAST [3]). We first test for incongruence between the timings of coalescent events, the clustering patterns and the known transmission events and links in `transmission unaware' analyses. Next, we explicitly test for a transmission-associated evolutionary rate decline using a mixed effects molecular clock model while imposing coalescent compatibility under the transmission model [4]. Finally, we estimate the contribution of the synonymous and non-synonymous components to the overall evolutionary rate at the within and between host levels.

Examination of the transmission chain data reveals several conflicts between the recovered timings and clustering patterns and the known transmission history, highlighting the need for incorporating host transmission information in the analysis. Like [1] we find a transmission-associated decrease in the evolutionary rate, and recover strong Bayes factor support for a lower rate on the transmitted lineages ($5.5x10^{-5}$ substitutions/site/year (95%HPD: 1.9-9.8)). In contrast with the larger effect on nonsynonymous substitutions reported by [1] we find an equal contribution of synonymous and nonsynonymous substitutions to the overall rate at both scales of evolution. We show that ignoring the tree structure biases the evolutionary rate estimates for a particular subgroup, which in turn leads [1] to flawed conclusions.

We recover comparably lower substitution rates at the between host level as [1]. Because these are still an order of a magnitude higher than the rate estimates obtained by calibrating the phylogeny using human-virus co-divergence data [5], this suggests that saturation and long-term purifying selection [6, 7] also contribute to masking the age of older coalescent events. A similar effect of transmission on synonymous and nonsynonymous substitutions suggests that the HBV within and between host evolutionary rate discrepancy is explained by a transmission/establishment advantage of variants that avoid accumulating mutations, perhaps by spending a larger part of their lifetime in the covalently closed circular DNA (cccDNA) state, rather than by the CAT model.

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65.

Oronasal immunization with low-passage MCMV HaNa1 in neonatal mice induces a strong protection against an infection with MCMV Smith in adulthood

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Human cytomegalovirus (HCMV) is an opportunistic herpesvirus, which infects approximately 50-100% of the adults worldwide. At present, there is no vaccine available against HCMV. A neonatal murine MCMV model was previously developed for a better understanding of natural primary HCMV infections in healthy infants. Here, we utilized this model to investigate whether oronasal immunization of neonatal mice with an MCMV strain gives a protection against an infection with another MCMV strain in adulthood. Mice were oronasally inoculated with 10^5 $TCID_{50}$ MCMV HaNa1 or PBS at 3 days of age and challenged with 10^5 $TCID_{50}$ MCMV Smith 10 weeks after the first infection. None of the mice showed clinical symptoms. At 7, 10, 14, 21 days post challenge (dpc), various tissues were collected for virus titration. Mandibular lymph nodes (LN), deep cervical LN and spleen of non-immunized mice were more swollen than that of HaNa1-immunized mice. In non-immunized mice, inoculation with MCMV Smith produced a typical systemic infection. Infectious virus was detected in nasal mucosa, lungs, spleen, submandibular glands, liver and kidneys. In contrast, only low levels of virus replication were detected in nasal mucosa of HaNa1-immunized mice at 7 dpc. In the other organs, no virus replication was detected. Cocultivation showed that cell-associated viremia was detected only in non-immunized mice. Whole IgG antibody and neutralizing antibody titers increased after challenge. Like the primary infection in neonatal life, the IgG isotype response to MCMV was still dominated by IgG2a after challenge. Furthermore, the MCMV-specific CD8 T cell response was boosted in NALT, deep cervical LN and spleen in HaNa1-immunized mice compared with non-immunized mice after challenge. Taken together, our results demonstrate that a protective immunity against an MCMV infection can be established during neonatal life. These results might bring new insights in the development of vaccine strategies against HCMV infection in humans.

66.

Effects of murid herpesvirus 4 infection on B-cell repertoire in mice

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To confer protection, the adaptive immune system produces a highly diversified repertoire of antibodies that are selected and expanded in response to specific antigens. While pathogens can affect the antibody repertoire of responding B cells, their effect on the whole repertoire is mostly unknown. This is especially true for persistent viruses such as Gammaherpesviruses (γHVs) which are ubiquitous in human and animal populations. Indeed, although γHVs induce a polyclonal B cell activation as a normal part of their life cycle, the consequences of these infections on the host's antibody repertoire are still largely unknown. Recent advances in high-throughput DNA sequencing technologies enabled characterization of the antibody repertoire. In this study, we used murid herpesvirus 4 (MuHV-4), a γHV infecting laboratory mice, to study the imprinting of a γHV infection on the antibody diversity of its host. Briefly, we developed in mice a consensus sequencing approach that incorporates unique barcode labels on each starting RNA molecules and therefore allows us to reduce rate of sequencing error and to quantify transcripts. Based on this technique, the subsequent bioinformatics analysis of antibody heavy chain sequences allowed us to compare the diversity, the isotype frequency, the level of somatic hypermutation and the lineage structure of the antibody repertoire in MuHV-4-infected and uninfected mice. Altogether, this study highlights that deep sequencing of immunoglobulin transcripts provides direct insight into the imprinting of γHVs infection on the immune system of their hosts.

67.

Establishment of Genital Mucosa Model to study Primary Invasion of BoHV-4

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Bovine herpesvirus 4 (BoHV-4) is a gamma herpesvirus that is widespread in cattle. An in vitro model of bovine genital tract mucosa explants was set up to study BoHV-4 cellular/molecular host-pathogen interactions. Bovine genital tract tissues including posterior vagina, cervix and uterus body tissues from cows in two reproductive cycle phases (luteal and follicular phase) were used for mucosa explants. Mucosa explants were maintained in culture for up to 96 hours without any significant changes in morphology and viability, as determined with a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining as a viability test. The replication kinetics and characteristics of BoHV-4 within the three different mucosae and two different reproductive cycle phases were assessed by virus titration of the supernatant, number of plaques, plaque latitude and number of infected cells. BoHV-4 replicated productively in all genital mucosal tissues**.** BoHV-4 epithelial plaques were formed in the genital tissues and did not cross the basement membrane. The plaque number and plaque latitude were higher in the luteal phase than in the follicular phase. Single infected cells were found in the lamina propria of the genital mucosa. The BoHV-4 infected cells were identified as cytokeratin$^+$ (epithelial) and CD172a$^+$ (monocyte/macrophage lineage) cells in the three parts of the genital tract at 24h pi. The in vitro study shows that the bovine genital tract explants are a valuable model for the investigation of the pathogenesis of BoHV-4.

68.

Cameroonian bats as reservoirs for novel highly divergent viruses

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Introduction

Over the last years, several viruses pathogenic to human are believed to have originated in bats, including Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS)-related coronaviruses, Ebola- and Marburgvirus, Nipah virus and Hendra virus. In some regions of Cameroon where bats are being hunted, traded on local markets and consumed, there is a considerable risk for zoonotic transmission between bats and humans. However, there is limited data available on the viruses that are circulating in Cameroonian bats and their potential to cause disease in humans. To investigate the zoonotic potential of bat viruses from Cameroon we investigated the fecal virome of the most prevalent bat species in Cameroon, the straw-colored fruit bat and the Gambian epauletted fruit bat which lives in close proximity with humans in Cameroon.

Methods

Fecal samples were collected from straw-colored and gambian epauletted fruit bats in the South West Region of Cameroon. Three to five samples were pooled (24 pools in total) and treated using the recently developed NetoVIR (Novel enrichment technique of VIRomes) protocol to enrich viral particles. Subsequently, nucleic acids were extracted, randomly amplified and sequenced using Illumina NGS technology. The resulting sequence reads were trimmed for quality and assembled *de novo* into contigs. Diamond was used to compare each contig against a non-redundant database containing all viral complete genomes for annotation purposes.

Results & Discussion

In total, 137 million reads were generated (± 9 million reads per pool). After filtering for bacterial reads and quality trimming, a total of 42 million reads were remaining of which 14% were of viral origin. Less than 5% of all reads belonged to viruses infecting vertebrates and are a possible causative agent for gastroenteritis in humans and included viruses belonging to the *Picornavirales* (*Fesavirus, Posavirus*, *Kunsagivirus*), *Caliciviridae (Sapovirus, Saporovirus), Reoviridae* (*Rotavirus A and H*), *Astroviridae*, *Picobirnaviridae, Circorviridae* and *Parvoviridae (Parvovirus* and *Densovirus)*. In addition, viruses with a high zoonotic potential belonging to the *Coronaviridae* (*Betacoronavirus*) or *Paramyxoviridae* (Nipah-like virus) families, as well as viruses belonging to families with a high cross-species barrier were detected (*Papillomaviridae* and *Herpesviridae)*. Sequence analysis of most of these viruses revealed a distant genetic relatedness to viruses present in databases. Phylogenetically, our bat viruses of the families *Caliciviridae*, *Astroviridae* and *Circoviridae* clustered (distantly) with viruses detected from human hosts, suggesting interspecies transmission in the distance past.

Conclusions

These results highlight the vast genetic diversity of viruses present in bats. Ongoing studies are: 1) screening more bat samples and 2) screening of samples of humans with gastro-enteritis living in the same area and in close proximity with these bats to investigate the potential role of bat viruses as etiology of episodes of human gastroenteritis.

69.

Gp150 promotes sexual transmission of Murid Herpesvirus-4.

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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 understand the mechanisms underlying natural transmission. Some of these mechanisms could rely on the glycoprotein 150 (gp150), which could limit virus neutralization and promote the release of infectious particles from 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 WT or gp150- strains expressing luciferase. They were imaged with an *in vivo* imaging system to follow infection. When lytic replication was observed 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 reaching and replicating in the female genital tract, it displays a major deficit of sexual transmission in comparison with WT virions. Interestingly, this deficit appears to reflect a deficit of virions release 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, by promoting the releasing of infectious particles from the mucosal cells.

70.

MCMV exploits the spleen as a transfer hub for systemic dissemination upon oronasal inoculation

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Abstract

Murine cytomegalovirus (MCMV) infection in mice is a commonly used animal model for studying human cytomegalovirus (HCMV) infection in humans. In our previous studies, a mouse model based on an oronasal MCMV infection was set up for mimicking a natural HCMV infection, and the spleen was hypothesized to contribute to viremia for virus dissemination to distal organs such as submandibular glands. Here, the role of the spleen in the natural MCMV infection was investigated in intact and splenectomized Balb/c mice with either MCMV Smith or MCMV HaNa1.

Various samples were collected at 7, 14 and 21 days post inoculation (dpi) for analysis. The results showed that for both virus strains, 1) cell-associated virus (determined by co-cultivation) in PBMC of splenectomized mice was undetectable compared with that of intact mice; 2) the mean of virus DNA load (determined by qPCR) in PBMC of splenectomized mice was significantly lower at peak viremia (7 dpi) in contrast to that of intact mice; 3) infectious virus (determined by virus titration) in the submandibular glands was detected later in splenecomized mice (14 dpi) than in intact mice (7 dpi). Moreover, the average virus titers in submandibular glands of splenectomized mice were around 8-10 times lower at 14 dpi and 1.6-2 times lower at 21 dpi compared with that of intact mice. Additionally, for the MCMV Smith strain, virus titers in the kidneys and liver of splenectomized mice were undetectable in comparison with intact mice. For MCMV HaNa1 strain, virus titers in the kidneys and liver were undetectable in both intact and splenectomized mice. Taken together, all these data clearly demonstrate that virus dissemination to other organs is reduced in splenectomized mice, indicating that the spleen serves as a viremia booming site for natural MCMV infection.

71.

Dual infections of equine herpesvirus 1 and equine arteritis virus in equine respiratory mucosa

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Equine herpesvirus 1 (EHV-1) and equine arteritis virus (EAV) induce respiratory problems and abortion in horses and are considered as two serious threats to equine industry. Both EHV-1 and EAV misuse patrolling leukocytes in the upper respiratory tract (URT) to breach the basement membrane (BM) and migrate to the blood vessels. So far, the behavior and impact of a double infection in the respiratory mucosa of a horse are unknown. In the present study, the outcome of double infections with EHV-1 and the low virulent EAV strain 08P187 (super-infections with an interval of 12h and co-infections) were compared with single infections in fully susceptible RK-13 cells and equine upper respiratory mucosa explants. When RK-13 cells were inoculated with either EHV-1 or EAV 12h prior to the subsequent EAV or EHV-1 inoculation, the latter EAV or EHV-1 infection was clearly suppressed at 24hpi or 36hpi, respectively, without EHV-1 and EAV co-infecting the same RK-13 cells. After simultaneous infection with EHV-1 and EAV, higher numbers of EAV infected cells but similar numbers of EHV-1 infected cells were found compared to the single infections, with a low number of EHV-1/EAV co-infected RK-13 cells from 48hpi. In the upper respiratory mucosa exposed to EAV 12h prior to EHV-1, the number and size of the EHV-1-induced plaques was similar to those of the EHV-1 single infected mucosa explants. In the lamina propria of nasal and nasopharyngeal mucosae, EAV and EHV-1 pre-infections slightly reduced the number of EHV-1 and EAV infected leukocytes compared to the single infections and co-infections. In double EAV/EHV-1 infected explants, no co-infected leukocytes were detected. From these results, it may be concluded that EAV and EHV-1 are only slightly influencing each other's infection and that they do not infect the same mucosal leukocytes.

72.

Inhibition of in vitro and in vivo Zika virus replication by the nucleoside analogue 7-deaza-2'C-methyladenosine.

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Zika virus (ZIKV) is a member of the flavivirus genus within the family of the Flaviviridae. The virus was first isolated in 1947 from a rhesus monkey in the Ugandan Zika Forest and is now widely prevalent in Africa and South East Asia and (more recently) the Americas, and it continues to spread. It is transmitted by mosquitoes, predominantly various *Aedes* species. ZIKV causes a disease called Zika fever that is characterized by headache, maculopapular rash, fever, malaise, conjunctivitis, and arthralgia. A ZIKV outbreak in 2007 in Micronesia resulted in about ~5000 infected individuals and during an explosive outbreak in French Polynesia in 2013 an estimated 28000 individuals sought medical care (~11% of the population). Currently, there is neither a vaccine, nor a specific therapy for the treatment of ZIKV infections.

After developing in-house in vitro antiviral assays (CPE-reduction assay, virus-yield reduction assay, immunofluorescence assay) for the screening of inhibitors against ZIKV and further confirmation of potential hits, a variety of known inhibitors of RNA viruses/flaviviruses were tested for their potential to inhibit ZIKV replication, including interferon-alpha, ribavirin, 2'-C-methylcytidine, 7-deaza-2'-C-methyladenosine (7'DMA), and favipiravir [T-705, a drug developed for treating influenza virus infections and recently shown to inhibit chikungunya virus replication] as well as its analogue T-1105. In particular, 7'DMA proved to be a selective and potent inhibitor of ZIKV replication with an EC_{50} value of 3-9 μg/ml. In order to evaluate its in vivo efficacy, we first established a robust ZIKV infection model in interferon α/β and γ receptor knock-out (AG129) mice. Intraperitoneal inoculation of 200-20000 PFU resulted in virus-induced disease (mean day of death 15 days post infection; MDD=15 days p.i.) with high virus titers detectable in serum and various organs (brain, spleen, liver and kidney). Infection resulted in the induction of various pro-inflammatory cytokines (IFN- γ, TNF-α, IL-6, IL-18) and chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CXCL1, CXCL2, CXCL10). Histopathological analysis further revealed the accumulation of viral antigens in the neurons of the brain and spinal cord. Acute neutrophilic encephalitis was observed at the time of onset of virus-induced morbidity. Treatment of ZIKV-infected AG129 mice with 7'DMA (50 mg/kg/dose, QD) significantly reduced viremia by 0.7-1.3$\log_{10}$ at day 5-8 p.i. compared to that of vehicle-treated mice. Moreover, the nucleoside analogue significantly delayed virus-induced mortality by 9 days (MDD = 14.5 and 23.5 days p.i. for vehicle- and 7'DMA-treated animals, respectively). 7'DMA also caused a lower induction of the pro-inflammatory cytokines IL-18 and IFN-γ.

In conclusion, we for the first time established a robust animal model of ZIKV infection with reported viremia, histopathological signs of acute neutrophilic encephalitis and pro-inflammatory cytokine and chemokine expression. Establishment of an animal model is essential for determining the in vivo efficacy of candidate molecules against ZIKV infection and, hence, will allow for a boost in antiviral drug development against this truly neglected tropical disease. A molecule that might serve as a possible lead candidate is the nucleoside analogue 7'DMA, which was found to efficiently inhibit ZIKV replication in vitro and in vivo.