2012

Confirmed speakers

Yoshihiro Kawaoka | Madison, USA

Jon McCullers | Memphis, USA

Joseph P. Mizgerd | Boston, USA

Juan Ortin | Madrid, Spain

Ron Fouchier | Rotterdam, The Netherlands

3rd International Influenza Meeting

FluResearchNet.

September 2nd – 4th

Organizing committee

Stephan Ludwig | Muenster

Klaus Schughart | Braunschweig

Peter Stäheli | Freiburg

Roland Zell | Jena

Friederike Jansen | Muenster

Isabell Schmid | Muenster

Venue:

University of Muenster

Schlossplatz 2

48149 Muenster

Germany

Nationale Forschungsplattform für Zoonosen

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Federal Ministry of Education and Research

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GENERAL INFORMATION

Scientific Committee
Stephan Ludwig, Münster
Klaus Schughart, Braunschweig
Peter Stäheli, Freiburg
Roland Zell, Jena

Offical Language
The official language of the meeting is English. Simultaneous translation will not be provided.

Poster Presentations
Posters are to be mounted between 15.00 and 21.00 p.m. on Sunday afternoon, September 2nd. Posters are to be removed between 14.00 and 17.00 p.m. on Tuesday, September 4th.

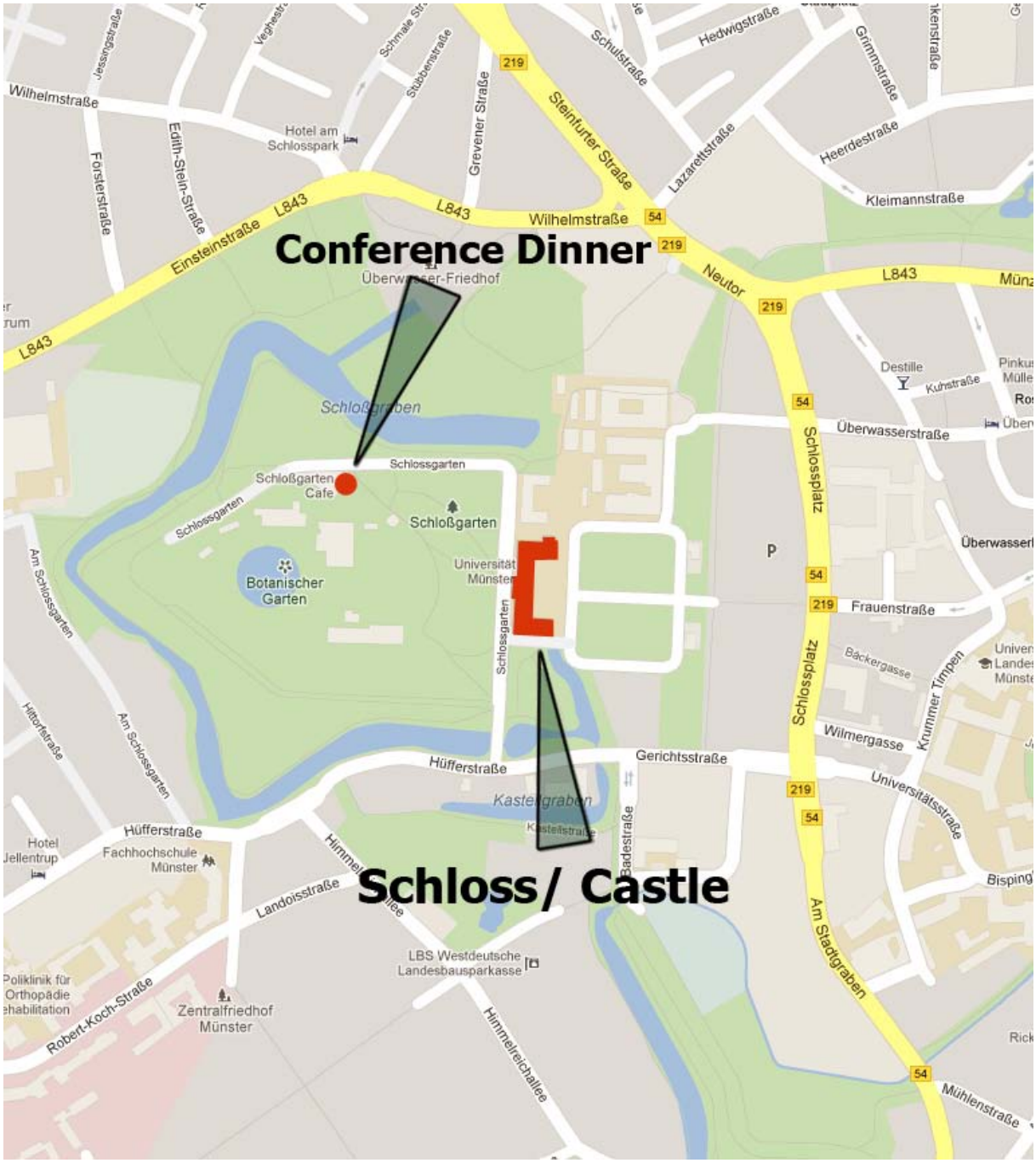
Meals
Lunches will be provided in the Foyer of the castle of Münster, as indicated in the program. Evening meals will be provided as part of the social program. You are invited by the organizers.

Social Program
The Opening reception will take place at the venue on Sunday, September 2nd and will start at 19.00 p.m.
Address: **Universität Münster,** Schlossplatz 2, D-48149 Münster

The Conference Dinner will take place at Schlossgarten Cafe. The restaurant is located in 2 minutes walking distance directly behind the venue – you will reach it by leaving the castle's backdoor and walking straight through the castle garden. The dinner starts at 19.00 pm.

Organization
FluResearchNet and National Platform for Zoonoses
c/o Institute of Molecular Virology (IMV)
Center of Molecular Biology of Inflammation (ZMBE)
Westfälische Wilhelms-Universität Münster
Von-Esmarch-Str. 56
D-48149 Münster
Phone: +49 (0)2 51/83 53011
Fax: +49 (0)2 51/83 57793
EMail: flu.2012@uni-muenster.de

VENUE


Conference Dinner
Schloss/ Castle
Wilhelmstraße
Einsteinstraße
L843
Steinfurter Straße
Hedwigstraße
Schulstraße
Grevener Straße
Hotel am Schlosspark
Forsterstraße
Edith-Stein-Straße
Lazarettstraße
Heerdestraße
Kleimannstraße
Neutor
Überwasser-Friedhof
Schloßgraben
Schlossgarten
Schloßgarten Cafe
Schloßgarten
Botanischer Garten
Universität Münster
Destille
Kuhstraße
Überwasserstraße
Schlossplatz
Frauenstraße
Bäckergasse
Krummer Timpen
Wilmergasse
Universitätsstraße
Gerichtsstraße
Hüfferstraße
Kastellgraben
Kastellstraße
Badestraße
Am Schlossgarten
Hittorfstraße
Hotel Jellentrup
Fachhochschule Münster
Landoisstraße
Himmelreichallee
LBS Westdeutsche Landesbausparkasse
Poliklinik für Orthopädie
Robert-Koch-Straße
Zentralfriedhof Münster
Am Stadtgraben
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ORAL PRESENTATIONS

Opening

Sunday, September 2nd 2012

Key-note lecture:

An epic journey to publish ferret H5N1 transmission studies
Yoshihiro Kawaoka, Madison, USA

Session 1

Pathogenesis I

Chair: Peter Stäheli

Monday, September 3rd 2012

Key-note lecture: Transmission of Influenza A/H5N1 Virus via Aerosol or Respiratory Droplets between Ferrets
Ron Fouchier, Rotterdam, Netherlands

Inhibition of Inflammosome Activation in Human Macrophages by Highly Pathogenic Avian Influenza Viruses Caused by Missing M2 Protein Expression
Judith Friesenhagen, Hannover, Germany

Viral Scission Machines: M2-mediated Influenza Virus Budding
Jeremy Rossman, Kent, United Kingdom

An Intermolecular RNA Interaction Required for Selective Packaging of the Segmented Influenza A Genome
Roland Marquet, Strasbourg, France

Inhibition of inflammasome activation in human macrophages by highly pathogenic avian influenza viruses caused by missing M2 protein expression

J. Friesenhagen[1,2*], Y. Boergeling[3], E. Hrincius[3], S. Ludwig[3] , J. Roth[2], D. Viemann[1]

[1]Medical School Hannover, Experimental Neonatology, Hannover, Germany; [2]University of Muenster, Institute of Immunology, Muenster, Germany; [3]University of Muenster, Institute of Molecular Virology, Muenster, Germany;

Infections of the human host by highly pathogenic avian influenza viruses (HPAIV) are characterized by development of a cytokine storm and systemic spreading of infection. Macrophages as part of the first barrier of defense against systemic infections are important producers of cytokines and therefore most likely to be involved in the emergence of a cytokine storm. Analyzing human blood-derived macrophages infected with low pathogenic human A/PR8/34 (H1N1) and HPAIVs A/FPV/Bratislava/79 (H7N7) and A/Thailand/1 (KAN-1)/2004 (H5N1) in a genome-wide microarray study, we found a surprisingly low inflammatory and antiviral response of macrophages infected with HPAIV in contrast to those infected with human H1N1. We were able to confirm these data in quantitative Real-Time-PCR experiments. Immunofluorescence and Western Blot analyses revealed that virus replication and expression of viral proteins in the host cell take place with one exception. Viral protein M2 is not expressed in macrophages in case of HPAIV infection. As M2 serves as second signal for inflammasome activation in macrophages, we found a significant reduction of IL-1β protein expression. Missing M2 expression therefore leads to subsequent suppression of an important part of the inflammatory and antiviral response and thus enables HPAIV to bypass effective immune responses of one of the most important cell types of the innate immune system. Consequently, systemic spreading of HPAIV infection is facilitated.

Key words: inflammatory response, innate immunity, cytokine storm, inflammasome, virus protein M2

Viral scission machines: M2-mediated influenza virus budding

J. Rossman[1*], G. Leser[2], R. Lamb[2]

[1]School of Biosciences, University of Kent, Canterbury, UK; [2]Howard Hughes Medical Institute, Northwestern University, Evanston, IL USA

While many of the interactions required for influenza virus assembly have been determined, the molecular machinery needed to complete the budding process has only recently been elucidated. We have found a new role for the influenza virus M2 protein in meditating virus budding, independent from its previously determined ion channel activity. Investigation into the role of the M2 protein showed that the protein possesses a well conserved amphipathic helix that is capable of altering membrane curvature in a cholesterol-dependent manner. In a reduced-cholesterol environment such as would be found at the neck of a budding virion, we see that M2 causes positive membrane curvature and scission, a process that is dependent on the M2 amphipathic helix. Utilizing reverse genetics to recover influenza virus containing a mutated amphipathic helix we see that the M2 amphipathic helix is necessary for membrane scission and for the release of budding virions. Further results show that the M2 protein from influenza B viruses is also capable of causing budding and membrane scission. We suggest that the M2 protein may mediate the final step of budding for all influenza viruses, serving as a virus-encoded scission machine.

Key words: budding, scission, M2

An intermolecular RNA interaction required for selective packaging of the segmented influenza A genome

R. Marquet[1*], C. Gavazzi[1], M. Yver[2], M. Rosa-Calatrava[2], B. Lina[2], C. Isel[1], and V. Moules[2]
[1]Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084 Strasbourg, France; [2]Virologie et Pathologie Humaine, Université Lyon 1, EA4610, Faculté de Médecine RTH Laennec, 69008 Lyon, France.

The segmented nature of the influenza A virus genome offers evolutionary advantages but complicates its packaging. Increasing evidence supports the existence of a selective packaging mechanism, but its molecular details remain elusive. Using an *in vitro* biochemical approach we identified, at the nucleotide level, an intermolecular interaction between two viral genomic RNA (vRNA) segments of an influenza A virus that involves regions not previously known to contain packaging signals. Viruses containing silent point mutations that disrupted this interaction *in vitro* or compensatory mutations that restore it were produced by reverse genetics. Mutations in one or the other interacting vRNAs significantly reduced viral replication, while compensatory mutations restored it to wild type levels, indicating that this vRNA/vRNA interaction also takes place in virions and/or in infected cells. Electron microscopy revealed a packaging defect in the mutant viruses, resulting in a ten-fold increase of empty viral particles. Competition experiments between wild type and mutant vRNAs revealed that influenza A viruses preferably package either two wild type or two mutant vRNAs, thus demonstrating a role for this vRNA/vRNA interaction in the selective packaging of the corresponding vRNPs. Our study suggests that selective packaging of the influenza A virus genome relies on an interaction network between the genomic RNA segments.

Key words: Influenza A, RNA packaging, vRNA, vRNP

Session 2

The NS1 Protein as a Modualtor of Cell Responses

Chair: Stephan Ludwig

Monday, September 3rd 2012

The NS1 Protein of Seasonal Influenza A Viruses Inhibits Virus- and IFN-dependent Induction of ISG15 in Human Cells
Jessica Knepper, Berlin, Germany

Influenza A Virus NS1 Induces Re-localisation of Cellular ADAR1 to Nucleoli During Infection
Artur Arikainen, Cambridge, United Kingdom

A Single Point Mutation (Y98F) Within the NS1 of Influenza A Viruses Dramatically Limits Lung Epithelial Cell Tropism and Virulence in Mice
Eike-Roman Hrincius, Muenster, Germany

Influenza B Virus NS1 Protein Controls Riplet-mediated RIG-I Activation
Matthias Budt, Berlin, Germany

The NS1 protein of seasonal influenza A viruses inhibits virus- and IFN-dependent induction of ISG15 in human cells

J. Knepper[1*], V. K. Weinheimer[1], T. Wolff[1]

[1]Div. of Influenza/Respiratory Viruses, Robert Koch Institute, Berlin, Germany

Influenza A virus (IAV) infection provokes an antiviral immune response including activation of IFN and IFN-stimulated proteins. One of these proteins is the Interferon-Stimulated Gene 15 (ISG15) product. ISG15 is a ubiquitin-like polypeptide that can be covalently attached to target proteins in a process mediated by the ligases Ube1L, UbcH8 and Herc5. The impact of ISG15 on IAV propagation as well as species specificity is subject of our investigation.
Our initial findings revealed a strong induction of ISG15 in A549 cell cultures upon infection with seasonal IAV. Interestingly, we observed on single cell level that this induction occurred predominantly in uninfected cells, whereas little ISG15 was detected in the initially infected cells. In contrast, a population of both infected and ISG15-positive cells was present after infection with a ΔNS1 mutant virus indicating a role for NS1 in suppressing ISG15 in the infected cells. This increased double-positive population was also observed upon infections with low-pathogenic avian IAV. Transfection-based experiments demonstrated that the presence of an active viral minigenome was sufficient to induce ISG15, which was inhibited by co-expressed viral NS1 protein. Furthermore, transfection of NS1 proteins of seasonal IAV remarkably reduced IFN-stimulated ISG15-induction. Knockdown of ISG15 in A549 cells had no effect on replication of seasonal IAV but led to enhanced replication efficiency in case of two low-pathogenic avian IAV.
In conclusion, our results indicate that the NS1 protein of human IAV suppresses not only virus- but also IFN-dependent ISG15-induction and imply a role of ISG15 in limiting IAV host range.

Key words: Influenza A, ISG15, species specificity

Influenza A virus NS1 induces re-localisation of cellular ADAR1 to nucleoli during infection

A. A. Arikainen[1*], Helen Wise[2], Julian Hiscox[3], Paul Digard[2]

[1]Division of Virology, Department of Pathology, University of Cambridge; [2]Roslin Institute, University of Edinburgh, UK; [3]University of Leeds, UK

The nucleolus is a dynamic hub of post-transcriptional RNA processing in eukaryotic cells. The influenza NS1 protein has previously been seen to localise to nucleoli, though its role there is not clear. We recently found that influenza A virus infection induces the re-localisation of adenosine deaminase acting on RNA 1 (ADAR1) to the nucleolus. ADAR1 is an RNA-editing enzyme that converts adenosine residues to inosine. There is potential that ADAR1 activity may affect the influenza virus genome; either negatively through hypermutation, or positively via a specific coding sequence alteration as seen with hepatitis delta virus. Evidence for non-specific editing of influenza RNAs has been reported, but the significance of this has not been demonstrated.

We find that NS1 is both necessary and sufficient to induce nucleolar re-localisation of ADAR1 in 293T, A549, HeLa and BHK cells. Analysis of viruses expressing mutant forms of NS1 showed that proteins with lesions in the RNA binding or TRIM25 interaction sites failed to induce this re-localisation. We also show by GFP-trap pull-down assays that ADAR1 interacts with influenza NS1 and the viral polymerase subunits in infected cells. However, virus replication in cells treated with siRNA against ADAR1 was reduced by 3-fold, not supporting the hypothesis that ADAR1 is a restriction factor for influenza A virus.

We conclude that the influenza A virus NS1 protein induces the nucleolar re-localisation of ADAR1 and that this interaction is of benefit to the virus. Further work is under way to test mechanistic hypotheses for how this might operate.

Key words: influenza, ADAR, nucleolus

A single point mutation (Y89F) within the non- structural protein 1 of influenza A viruses dramatically limits lung epithelial cell tropism and virulence in mice.

Eike R. Hrincius[1*], Ann-Katrin Henneke[1], Lisa Gensler[1], Darisuren Anhlan[1], Peter Vogel[2], Jon McCullers[3], Stephan Ludwig[1] and Christina Ehrhardt[1]

[1] Institute of Molecular Virology (IMV), ZMBE, Von Esmarch-Str. 56, D-48149 Muenster, Germany
[2] St. Jude Children's Research Hospital, Veterinary Pathology, 262 Danny Thomas Place, Memphis, TN 38105-3678
[3] St. Jude Children's Research Hospital, Department of Infectious Diseases, 262 Danny Thomas Place, Memphis, TN 38105-3678

The non-structural protein 1 (A/NS1) of influenza A viruses (IAV) harbors several *src homology domain* (SH)-binding motifs (bm) (one SH2bm and two SH3bm), which mediate interaction with cellular proteins. In contrast to the sequence variability of the second SH3bm, the tyrosine 89 within the SH2bm is highly conserved among different IAV strains. This prompted us to evaluate the necessity of this SH2bm for IAV virulence. In an *in vivo* mouse model, we observed a dramatically reduced body weight-loss and reduced mortality upon infection with the A/NS1 Y89F mutant in comparison to wild-type virus. Infectious titers in the lung and bronchoalveolar-lavage fluid (BALF) were also reduced in comparison to wild-type virus. Concomitantly, we observed decreased cytokine, chemokine and immune cell levels in the lung and BALF as well as less severe pathological changes, reflecting reduced levels of virus-titers. Interestingly the replication of the A/NS1 mutant in mouse lung was overall reduced and strongly restricted to alveoli and if any marginally to bronchioli. In contrast, wild-type virus infection led to virus antigen positive areas in tracheal, bronchus, bronchiole and alveolar epithelium. Finally, wild-type virus infection resulted in a dramatic destruction of the bronchiole epithelium in clear contrast to infection with the A/NS1 mutant. Here, a slightly hypertrophic but entirely intact bronchiole epithelium was observed.
Taken together, we could show that disruption of the highly conserved SH2bm within the A/NS1 results in decreased virus distribution in the mouse lung and dramatically reduces virulence illustrating the necessity of the SH2bm for IAV induced pathogenicity.

Key words: influenza A virus, NS1 protein, SH binding motif, virulence, immune response

Influenza B Virus NS1 protein controls Riplet-mediated RIG-I activation

M. Budt[1*], R. Daviña Núñez[1], T. Wolff[1]

[1] Robert Koch Institute, FG17 – Influenza / Respiratory Viruses
Nordufer 20, D-13353 Berlin, Germany

The retinoic acid inducible gene I product (RIG-I) functions as a cellular sensor detecting viral 5´-PPP-RNA during influenza virus infection and triggering the antiviral I interferon response. RIG-I activity is tightly controlled by ubiquitination, mediated by at least three ubiquitin ligases. Previously, it was shown, that the influenza A virus NS1 protein inhibits RIG-I activation by interfering with its TRIM25-mediated ubiquitination.
Here, we elucidated the molecular mechanism on how the highly divergent influenza B virus blocks the antiviral response. Using RNAi we show that RIG-I is essential for influenza B virus induced IFNβ production. Reporter assays of transiently transfected cells revealed that the E3 ligase Riplet (RNF135/REUL) has a very strong impact on RIG-I induced IFNβ promoter activity and enhanced RIG-I activation by influenza virus RNA. Riplet stimulates covalent ubiquitination of RIG-I in a manner dependent on lysine 63 of ubiquitin. Consistently, mutation of key lysine residues in RIG-I abrogated its activation by viral RNA or Riplet. The influenza B virus NS1 protein (B/NS1) interacts with RIG-I and Riplet in bimolecular fluorescence complementation assays. B/NS1 inhibits the RIG-I-dependent induction of the IFNß promoter both in infected and transfected cells. The IFN-inhibitory function was mapped to the C-terminal region of B/NS1.
In conclusion, our data suggest that B/NS1 inhibits the RIG-I dependent signalling pathway by binding to Riplet acting upstream of RIG-I and thus executing an important pathogen-host interaction in the influenza virus replication cycle.

Key words: RIG-I, Type I Interferon, ubiquitination, Riplet, NS1

Session 3

Innate Immunity

Chair: Martin Schwemmle

Monday, September 3rd 2012

Inhibition of Influenza Virus Infectivity In Vivo Using RNA Agonists of the RIG-I Pathway
John Hiscott, Montreal, Canada

Incoming Influenza A Virus Evades the Early Host Recognition – Direct Interferon Induction by Influenza B Virus Entry
Pamela Österlund, Helsinki, Finland

Evolution-guided Analysis of Human and Non-human Primate MxA Proteins: A Motif in Unstructured Loop L4 is Essential for Antiviral Specificity Against Orthomyxoviruses
Corinna Patzina, Freiburg, Germany

The Mx System of Wild-derived CAST/EiJ Mice Confers Different Degrees of Protection Against Orthomyxoviridae Family Members
Cindy Nürnberger, Freiburg, Germany

Inhibition of influenza virus infectivity *in vivo* using RNA agonists of the RIG-I pathway

John Hiscott[1, 2*], Marieline Goulet[2], Zheng-Yun Xu[2], David Olagnier[1], Zhong He[1], Suzanne Paz[2], Meztli Arguello[2], and Rongtuan Lin[2]

[1]Vaccine & Gene Therapy Institute of Florida, Port St. Lucie FL
[2]Lady Davis Institute-Jewish General Hospital, McGill University, Montreal, Canada

With new antigenic strains of influenza continuing to emerge, rapid generation of effective vaccines remains a challenge, and antigen-independent prophylaxis may provide an alternative approach to enhance host resistance against infection. The characterization of agonists that potently activate the innate immune system, trigger the host antiviral response and inhibit H1N1 Influenza virus infection are potentially useful in limiting virus multiplication *in vivo* and may also serve as adjuvants in vaccine development. The cytosolic RIG-I pathway is activated by many RNA viruses - including influenza - via viral RNA replicative intermediates that contain short hairpin dsRNA and 5' triphosphate structures. We characterized natural and synthetic RIG-I agonists based on sequences from the 5'UTR regions of distinct negative-strand viruses – vesicular stomatitis virus (VSV), Influenza, Rabies, Measles, and Sendai virus - and demonstrated potent stimulation of RIG-I antiviral responses at concentrations in the picomolar range. In human bronchial epithelial A549 cells, 5'pppRNA induced IRF3 phosphorylation and dimerization, STAT1 Tyr701 phosphorylation, as well as a >100-fold increase in the transcription of interferon stimulated genes (ISGs) and genes involved in inflammation. The magnitude and duration of ISG and inflammatory gene expression was evaluated by gene expression profiling, where 5'pppRNA triggered a sustained and diverse range of antiviral and inflammatory genes compared to treatment with IFNα, and bioinformatics analysis identified distinct nodes of IRF7, IRF1 and NF-□B gene activation. Pre-treatment of A549 cells with 5'pppRNA dramatically blocked H1N1 A/PR/8/34 Influenza virus replication; furthermore, intravenous delivery of 5'pppRNA to BALB/c mice generated an antiviral response in mouse lungs that protected animals from a lethal challenge with H1N1 A/PR/8/34. RNA agonist delivery inhibited virus replication in mouse lungs within the first 24h after H1N1 challenge and protected the lungs of infected animals from virus-induced pathology. Finally, 5'pppRNA pre-treatment also completely or partially blocked replication of Dengue, Vaccinia and HIV-1 replication. These results illustrate that naturally derived RIG-I agonists represent a potent stimulator of the innate antiviral response, with the capacity to block replication of multiple pathogenic human viruses.

Incoming Influenza A Virus Evades the Early Host Recognition – Direct Interferon Induction by Influenza B Virus Entry

P. Österlund[1*], M. Strengell[1], L.P. Sarin[2,3], M.M. Poranen[3], R. Fagerlund[1], K. Melén[1], I. Julkunen[1]

[1]National Institute for Health and Welfare, Virology Unit, Helsinki, Finland; [2]University of Helsinki, Department of Biosciences and [3]Institute of Biotechnology, Helsinki, Finland

Activation of interferon (IFN) system, which is triggered largely by the recognition of viral nucleic acids, is one of the most important host defense reactions against viral infection. Although influenza A and B viruses, which both have segmented negative strand RNA genome, share major structural similarities, they have evolutionary diverged with total genetic incompatibility. We compared antiviral-inducing mechanisms during the infection with type A and B influenza viruses in human dendritic cells. We observed that IFN responses are induced significantly faster in cells infected with influenza B virus than type A virus, and that the early induction of antiviral gene expression is mediated by the activation of transcription factor IRF3. We further demonstrate that influenza A virus infection activates the IFN responses only after the viral RNA (vRNA) synthesis, whereas influenza B virus induces IFN responses even if its infectivity is destroyed by UV treatment. Thus, initial viral transcription, replication and viral protein synthesis are dispensable for the influenza B virus-induced antiviral responses. Moreover, viral ribonucleoprotein (vRNP) complexes and vRNA molecules from both type A and B virus are equally potent activators of the IFN induction, but, instead, incoming influenza B virus structures are recognized directly in the cytosol, while influenza A virus is able to evade the early recognition. Collectively, our data provide new evidence of a novel antiviral evasion strategy for influenza A virus without contribution of viral NS1 protein and this opens up new insights into different influenza virus pathogenicity.

Key words: Influenza A and B, Interferon, virus recognition, host responses

Evolution-guided analysis of human and non-human primate MxA proteins: a motif in unstructured loop L4 is essential for antiviral specificity against orthomyxoviruses

Corinna Patzina[1*], Patrick S. Mitchell[2], Michael Emerman[2], Otto Haller[1], Harmit S. Malik[2], Georg Kochs[1]

[1]Department of Virology, University of Freiburg, Germany; [2]Fred Hutchinson Cancer Research Center, Seattle, USA

Influenza virus replication is potently inhibited by host restriction factors, e.g. the IFN-inducible myxovirus resistance proteins (MxA). While human MxA (hsMxA) is a well-studied antiviral effector protein, the antiviral function of closely related MxA orthologs of non-human primates is so far unknown. Thus, we characterized the activity of a set of primate MxA proteins against hsMxA-sensitive viruses. Surprisingly, the orthomyxoviruses influenza A and Thogoto virus (THOV) were restricted mainly by hominoid MxA proteins but not by gibbon, old or new world monkey MxA proteins.

We further performed an evolution-guided analysis determining motifs in MxA under recurrent positive selection, as a proposed signature of host-pathogen interfaces. Thereby, we identified unstructured loop 4 (L4) as the most divergent moiety of MxA. MxA GTPases consist of an N-terminal globular G-domain and a C-terminal stalk with effector functions from which the flexible, surface-exposed L4 is protruding. By replacing primate MxA L4 with its human counterpart, we created chimeric proteins that gained activity against influenza A and THOV, thus changing their antiviral profile to that of hsMxA. Further analysis revealed that a single site in L4 under positive selection is essential for MxA activity against orthomyxoviruses, demonstrating that minimal changes in L4 determine MxA antiviral specificity.

In summary, functional and phylogenetic analyses of primate MxA proteins revealed a distinct species-specific antiviral profile and identified L4 as the putative interface required for activity against orthomyxoviruses, most likely by directly interacting with viral ribonucleoprotein complexes as the target of antiviral action.

Key words: innate immunity, orthomyxovirus, primate MxA

The Mx system of wild-derived CAST/EiJ mice confers different degrees of protection against *Orthomyxoviridae* family members

C. Nürnberger[1,2*], K. Friedrich[1], M.H. Christensen[1], M.T. Ferris[3] , P. Staeheli[1]

[1]Dept. Virology, University of Freiburg; [2]Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg; [3]Dept. Genetics, University of North Carolina-Chapel Hill

Myxovirus resistance (Mx) proteins are interferon-induced dynamin-like GTPases exhibiting antiviral activity in many vertebrates. Murine Mx1 plays a crucial role in protection against orthomyxoviruses such as influenza A and B viruses, and Thogoto virus (THOV). Mice with functional Mx1 alleles are highly resistant to these viruses, whereas standard inbred mice which lack functional Mx1 succumb to lethal infection. Wild mouse strains possess a second Mx gene (Mx2) which is also defective in standard laboratory mice. Unlike Mx1 which shows nuclear localization and protects against specific viruses with nuclear replication site, Mx2 accumulates in the cytoplasm and has been shown to inhibit certain viruses replicating within this cell compartment.

We observed that CAST/EiJ inbred mice which are derived from the subspecies *Mus musculus castaneus* readily succumb to influenza A virus infection but are highly resistant to THOV infection. Sequence analysis revealed that these mice code for a full-length Mx1 protein which differs only in two amino acids from the well-characterized Mx1 protein of A2G mice. CAST/EiJ mice further contain an intact open reading frame for Mx2 lacking one amino acid compared to Mx2 of wild mice. Upon interferon stimulation, both Mx1 and Mx2 genes are expressed in embryonic fibroblasts derived from CAST/EiJ mice.

We are currently investigating if the amino acid changes in CAST Mx1 might reduce GTPase activity which could preferentially affect its anti-flu activity. Alternatively, we consider that Mx1 in CAST/EiJ mice is inactive and that the available Mx2 confers THOV resistance in these mice.

Key words: Mus musculus castaneus, Myxovirus resistance genes, interferon, influenza A virus, Thogoto virus

Session 4

Pathogenesis II

Chair: Klaus Schughart

Monday, September 3rd 2012

Key-note lecture: Structural and Functional Studies on the Influenza Virus Replication Machine
Juan Ortin, Madrid, Spain

The Avian-like PB1 Gene of the 1968 Pandemic Influenza Virus Facilitates Viral Replication and Transmissibility
Isabel Wendel, Marburg, Germany

The Assembly Mechanism of Influenza A Virus Nucleoprotein in Viral Ribonucleoprotein Complexes
Frank Vreede, Oxford, United Kingdom

Mapping the Phosphoproteome of Influenza A and B Viruses by Mass Spectrometry
Edward Hutchinson, Oxford, United Kingdom

Influenza A Virus Replication Can Be Prevented by Inhibition of Viral Ribonucleoproteins Transport
Maria Amorim, Oeiras, Portugal

The avian-like PB1 gene of the 1968 pandemic influenza virus facilitates viral replication and transmissibility

I. Wendel[1*], D. Rubbenstroth[2], P. Stäheli[2], H.D. Klenk[1], M. Matrosovich[1]

[1]Institute of Virology, University of Marburg, Marburg, Germany; [2]Department of Virology, University of Freiburg, Freiburg, Germany

The 1957 and 1968 pandemic influenza viruses emerged through reassortment between avian and contemporary human influenza viruses and contained HA and PB1 genes of avian origin. Acquisition of the avian HA allowed pandemic viruses to escape immune response in the human population; the role of the avian PB1 remains unknown. To address this long-standing question, we modelled the emergence of the 1968 pandemic H3N2 virus from its human H2N2 precursor. Based on analysis of publicly available sequences, A/California/1/66 (H2N2) (Cal66) was used as the putative human precursor. A/Hong Kong/1/68 (HK68) represented the H3N2 pandemic virus. A comparison of polymerase complexes of Cal66, HK68 and their reassortants using minigenome reporter assays demonstrated enhanced polymerase activity of complexes with avian-like PB1 of HK68. We next generated two recombinant Cal66 virus pairs, in which either the PB1 segment alone or both the PB1 and HA segments of Cal66 were substituted by corresponding segments of HK68. Using three different viral replication assays, we found that the virus with PB1 and HA of HK68 replicated more efficiently in human cells than its counterpart with PB1 of Cal66. This virus also transmitted more efficiently in guinea pigs. Our data demonstrate for the first time that avian-like PB1 of the 1968 pandemic virus served to promote viral growth and transmission.

Key words: influenza, pandemics, PB1, replication efficiency, transmission in guinea pigs

The assembly mechanism of influenza A virus nucleoprotein in viral ribonucleoprotein complexes

Lauren Turrell[1], Jon W. Lyall[2], Laurence S. Tiley[2], Ervin Fodor[1], Frank T. Vreede[1*]

[1]Sir William Dunn School of Pathology, University of Oxford, OX1 3RE, UK; [2]Department of Veterinary Medicine, University of Cambridge, Cambridge, CB3 0ES, UK

Each genomic RNA segment (vRNA) of influenza virus is associated with multiple copies of the nucleoprotein (NP) and a heterotrimeric RNA-dependent RNA polymerase (RdRp) to form the viral ribonucleoprotein (vRNP) complex that is responsible for viral transcription and replication. NP represents the structural scaffold for the vRNP and has been shown to engage in essential interactions both with neighbouring NP molecules through homo-oligomerisation and with the PB1 and PB2 subunits of the polymerase. NP also has inherent ssRNA-binding properties but with little or no sequence-specificity. The precise role of NP in RNP assembly and activity is not yet clear, due largely to its indispensability for vRNP activity. We have developed assays for the *in vivo* replication and transcription of vRNA-like templates by viral RdRp independent of NP or supported by the presence of homo-oligomerisation mutants of NP. Here, we demonstrate that homo-oligomerisation of NP represents the mechanism for a “tail-loop first” directional assembly of NP onto nascent RNP complexes, independent of and prior to RNA binding. We also find that the RNA-binding activity of NP is critical to its function during replication, whereas transcription is stimulated by the presence of RNA-binding mutants of NP. This work provides new mechanistic insights into the assembly and regulation of viral RNPs.

Key words: ribonucleoprotein complex, nucleoprotein, assembly, replication and transcription

Mapping the Phosphoproteome of Influenza A and B Viruses by Mass Spectrometry

E.C. Hutchinson[1*], E.M. Denham[1], B. Thomas[1], D.C. Trudgian, S. Hester[1], G. Ridlova[1], L. Turrell[1] and E. Fodor[1]

[1]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Phosphorylation is a common regulatory post-translational protein modification in eukaryotic cells. We used mass spectrometry to search for phosphorylated residues in all the proteins of both influenza A and B viruses – to the best of our knowledge, the first time such a comprehensive approach has been applied to any virus.

We detected phosphorylation of PB1, HA, NP, NA, M1, M2, NS1 and NEP, identifying 19 novel phosphorylation sites and confirming previously-identified sites. N-terminal processing of proteins was also detected. The structural context these sites suggests roles for phosphorylation in regulating activity (of HA and NA), nuclear transport (of M1, NP, NS1 and, through NP and NEP, of the viral RNA genome) and protein complex assembly (of NS1 dimers, M2 tetramers and NP oligomers). Mutational analysis of phosphorylation sites in NP was carried out, showing that phosphorylation sites in the N-terminal nuclear localisation signal are important for viral growth, whereas mutating sites in the C-terminus has little or no effect. Mutating phosphorylation sites in the oligomerisation domains of NP has an intermediate effect, and suggests that constitutive phosphorylation is not optimal for the virus. The conservation of phosphorylation sites in M1, M2 and NEP across different influenza virus genera suggests a fundamental role for phosphorylation in the lifecycles of all influenza viruses.

Taken together, the conservation and functional significance of phosphorylation sites implies a key role for phosphorylation in influenza biology, and also suggests a range of potential antiviral targets.

Key words: phosphorylation, mass spectrometry, nucleoprotein, trafficking, assembly

Influenza A virus replication can be prevented by inhibition of viral ribonucleoproteins transport.

Maria Joao Amorim[1,*], Richard Kao[2] and Paul Digard[3]

[1]Instituto Gulbenkian de Ciencia, Oeiras, Portugal
[2]Research Center of Infection and Immunity, the University of Hong Kong, Hong Kong
[3]Roslin Institute, University of Edinburgh, Scotland, UK

Influenza A virus (IAV) infects millions of people yearly, causing serious epidemics and in rare cases pandemics. Novel antivirals are needed to supplement existing control strategies. A promising new class of drug, exemplified by the compound nucleozin, has recently been identified that targets the viral nucleoprotein (NP). Based on structural and biophysical analyses, these inhibitors are thought to act as 'molecular staples' that stabilise interactions between NP monomers, promoting the formation of non-functional aggregates. Here we report an analysis of the inhibitory mechanism of nucleozin in virus infected cells. We find that the drug has both early- and late-acting effects on the IAV lifecycle. When present at the start of infection, it inhibits viral RNA synthesis, most likely by blocking entry of incoming genomic ribonucleoproteins (RNPs) into the nucleus. When added at later time points, it potently blocked the production of infectious progeny without affecting viral macromolecular synthesis. Instead, nucleozin promoted the aggregation of circulating RNPs and cellular Rab11, that had undergone nuclear export. We conclude that the primary target of nucleozin is the viral RNP, not NP and this work also provides proof of principle that IAV replication can be effectively inhibited by blocking trafficking of the viral genome.

Session 5

Influenza and the Lung

Chair: Thorsten Wolff

Tuesday, September 4th 2012

Key-note lecture: Cell-specific Innate Immunity During Lung Infection
Joseph P. Mizgerd, Boston, USA

Alveolar Epithelial Cells Orchestrate Dendritic Cell Functions by Release of GM.CSF in Murine Viral Pneumonia
Susanne Herold, Gießen, Germany

The Role of Alveolar Type II Epithelial Cells and TLR7 for Bacterial Superinfection Following Influenza
Sabine Stegemann-Koniszewski, Braunschweig, Germany

Deep Sequencing of Viral Quasispecies of Mammalian Adapted and Aerosol Transmitted Highly Pathogenic Avian Influenza A H5N1 Virus Over Time
Sander van Boheemen, Rotterdam, Netherlands

Alveolar epithelial cells orchestrate dendritic cell functions by release of GM-CSF in murine viral pneumonia

Barbara Unkel[1], Katrin Hoegner[1], Björn E. Clausen[2], Peter Lewe-Schlosser[3], Johannes Bodner[4], Stefan Gattenloehner[5], Hermann Janßen[6], Werner Seeger[1], Juergen Lohmeyer[1], and Susanne Herold[1*]

[1]Department of Internal Medicine II, [3]Center for Radiation Therapy, [4]Department of General and Thoracic Surgery, [5]Department of Pathology, University of Giessen and Marburg Lung Center (UGMLC), Member of the German Lung Research Center (DZL), Giessen, Germany
[2]Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, Netherlands, [6]Department of Surgery II, Hospital Dueren, Dueren, Germany

Influenza viruses (IV) cause pneumonia in humans with progression to lung failure. Pulmonary dendritic cells (DC) are key players in the anti-viral immune response, which is crucial to restore alveolar barrier function. However, the mechanisms of expansion and activation of pulmonary DC populations in lung infection remain widely elusive. Using mouse bone marrow chimeric and cell-specific depletion approaches, we demonstrate that alveolar epithelial cell (AEC) GM-CSF (granulocyte-colony stimulating factor) mediates recovery from IV-induced injury by affecting lung DC functions. Epithelial GM-CSF induced the recruitment of $CD11b^+$ and monocyte-derived DC and was required for the presence of $CD103^+$ DC in the lung parenchyma at baseline and for their sufficient activation and migration to the draining mediastinal lymph nodes (MLN) during IV infection. GM-CSF-dependently expanded and activated $CD103^+$ DC were indispensable for sufficient clearance of IV by antigen-specific $CD8^+$ T cells and for recovery from IV-induced lung injury. Moreover, intratracheally applied GM-CSF activated $CD103^+$ DC for increased migration to MLN and enhanced viral clearance, resulting in attenuated lung injury. Altogether, our data reveal a novel GM-CSF-dependent cross-talk between IV-infected AEC and $CD103^+$ DC which is crucial for effective viral clearance and recovery from injury with potential implications for treatment of severe IV pneumonia.

Key words: influenza, GM-CSF, dendritic cells, lung injury, alveolar epithelial cells

The role of alveolar type II epithelial cells and TLR7 for bacterial superinfection following influenza

S. Stegemann-Koniszewski[1,2*], S. Orrskog[3], M. Gereke[1,4], B. Henriques-Normark[3], M. Gunzer[2], D. Bruder[1,4]

[1]Helmholtz Centre for Infection Research Braunschweig; [2]University Duisburg-Essen; [3]Karolinska Institute Stockholm; [4]University Magdeburg

Influenza A virus (IAV) infections often predispose patients for disease through secondary bacterial pathogens such as *Streptococcus pneumoniae* by modulating anti-bacterial defense. This phenomenon poses a serious threat to human health and substantially contributed to morbidity and mortality during past influenza pandemics. Despite growing insight into the mechanisms of the viral/bacterial synergism, we are lacking a full understanding of the underlying triggering events. In a mouse co-infection model we analyzed the role of the pattern-recognition receptor (PRR) Toll-like receptor 7 (TLR7) in anti-influenza responses and secondary pneumococcal disease.

Microarray analyses of primary alveolar type II epithelial cells isolated from IAV infected mice early following infection revealed strong anti-viral and inflammatory responses which were attenuated in TLR7ko cells. Additionally, we found that TLR7 significantly contributes to the early airway interferon-gamma response conferred by NK cells following IAV infection. Nevertheless, TLR7-deficient hosts showed unchanged viral clearance and survival. In secondary pneumococcal infection, systemic dissemination of bacteria was significantly delayed in TLR7ko compared to wild-type mice while their alveolar macrophages showed increased phagocytic function. However, absence of TLR7 was not able to protect from the dramatically increased mortality to infection with a low-dose of pneumococci when it was preceded by IAV infection.

We present a new concept where a virus-sensing PRR influences defense towards a secondary bacterial pathogen. At the same time, the unchanged fatal overall outcome of co-infection in TLR7ko and wild-type hosts once more highlights the complexity of the factors involved in the synergism between viral and bacterial pathogen

Key words: influenza, bacterial superinfection, Toll-like receptor 7, alveolar type II epithelial cells

Deep sequencing of viral quasispecies of mammalian adapted and aerosol transmitted highly pathogenic avian influenza A H5N1 virus over time

Sander van Boheemen[1*], Sander Herfst[1], Martin Linster[1], Eefje J.A. Schrauwen[1], Theo M. Bestebroer[1], Guus F. Rimmelzwaan[1], Albert D.M.E. Osterhaus[1], Ron A.M. Fouchier[1]

[1]Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands

Highly pathogenic avian influenza A/H5N1 viruses continue to circulate in poultry in countries in Asia, Africa, and the Middle East. From 2003 through 12 April 2012, 602 laboratory-confirmed cases of A/H5N1 virus infections in humans were reported to WHO from 15 countries, but sustained transmission between humans has not yet been reported. More research is needed to determine the risk of mammalian adaptation and aerosol or respiratory droplet transmission of A/H5N1 virus.
Recently, we have demonstrated that A/H5N1 virus can acquire the ability of aerosol or respiratory droplet transmission between ferrets. Such transmission of A/H5N1 virus occurred upon targeted mutagenesis and serial virus passage in ferrets.
Here, we used deep sequencing on a 454-platform to monitor the emergence of viral quasispecies over the serial virus passages in these ferret experiments. We first compared quasispecies emergence by full-genome sequencing using a selection of nasal turbinates, nasal swabs, nasal washes, lungs, and throat swabs upon passaging of a wild-type and a genetically modified A/H5N1 virus. We then selected mutations of interest for further deep sequencing of all virus passages and all aerosol-transmitted viruses to monitor emergence of the virus variants over time.
This research describes the genetic changes over time of A/H5N1 wild-type and mutant viruses in a mammalian host, and monitoring the genetic changes associated with aerosol transmission. We conclude that such genetic changes occurred relatively rapidly in the ferret model.

Key words: transmissible H5N1, deep sequencing, mammalian adaptation, quasispecies

Session 6

Vaccines & Antivirals

Chair: Stephan Pleschka

Tuesday, September 4th 2012

Induction of Cross-Clade Anti-H5N1 Memory Responses by Vero Cell-Derived H5N1 Whole Virus Vaccines
Otfried Kistner, Orth/Donau, Austria

M2e-based Universal Vaccine Protects Against Influenza A Virus Challenge and Enhances Heterosubtypic Immunity During Subsequent Infections
Michael Schotsaert, Ghent, Belgium

VSV Replicons Expressing H5 Hemagglutinin Induce Broadly Neutralizing Antibodies Directed Against Both the HA1 and HA2 Subunit
Stefan Halbherr, Mittelhäusern, Switzerland

Antivial Activity of Ladania067 An Extract from Ribes Nigrum Against Influenza A Virus
Emanuel Haasbach, Tübingen, Germany

M2e-based universal vaccine protects against Influenza A virus challenge and enhances heterosubtypic immunity during subsequent infections

A. Michael Schotsaert [1,2*] , B. Tine Ysenbaert [1,2] , C. Katrijn Neyt [1,2] , D. Lorena Itati Ibanez [1,2], E. Pieter Bogaert [1,2] , F. Bart Lambrecht [1,2,3] , G. Walter Fiers [1,2] , H. Xavier Saelens [1,2]

[1] Department for Molecular Biomedical Research, VIB, 9000 Ghent, Belgium
[2] Department of Biomedical Molecular Biology, Ghent University, 9000 Ghent, Belgium
[3] Department of Respiratory Diseases, University Hospital Ghent, 9000 Ghent, Belgium

Influenza is responsible for half a million deaths and several million severely ill subjects, yearly. Influenza vaccines protect by raising antibodies that potentially neutralize the virion. However, due to sequence drift or shift, the virus escapes neutralizing immunity. Infection with a seasonal influenza virus correlates with protection against a subsequent infection with a heterotypic or heterologous virus, and this heterosubtypic immunity (HSI) very often is accompanied by the activation of an antiviral T-cell response. Vaccination with licensed inactivated vaccines protects against a homologous infection but also impairs the induction of cellular HSI. We demonstrated that Matrix 2 protein ectodomain (M2e)-based vaccines not only protect against influenza A virus challenge but also allow the induction of cellular HSI in mice. Vaccination with a recombinant M2e-virus like particle (M2e-VLP) strongly reduced morbidity and virus replication after a sublethal H3N2 infection and protects from mortality following a lethal challenge with pandemic H1N1 virus. Vaccination with whole inactivated virus (WIV) also protected mice against the homologous sublethal H3N2 infection but failed to protect against a subsequent lethal heterologous pandemic H1N1 challenge. Contrary to vaccination with WIV, vaccination with M2e-VLP followed by a sublethal infection with H3N2 virus resulted in the induction of a qualitative and functional cellular immune response and in the induction of iBALT. We hereby showed that, contrary to vaccination with WIV, vaccination with M2e-VLP universal vaccine does not only protect against challenge with viruses from different subtypes, but also permits the induction of cellular immunity induced by encounters with influenza virus.

Key words: Influenza, matrix 2 protein, universal vaccine, heterosubtypic immunity

VSV replicons expressing H5 hemagglutinin induce broadly neutralizing antibodies directed against both the HA_1 and the HA_2 subunit

Stefan Halbherr*, Merve Aricibasi, Terza Brostoff, & Gert Zimmer

Institut für Viruskrankheiten und Immunprophylaxe (IVI), CH-3147 Mittelhäusern, Switzerland

The rapid antigenic drift of influenza viruses is a major hurdle for the generation of vaccines providing durable protection. Most neutralizing antibodies interfere with the receptor-binding activity of HA and bind to epitopes in the globular head within the HA_1 subunit. These epitopes are primarily subject to antigenic drift. However, antibodies directed to the conserved HA_2 subunit may have neutralizing activity as well. They interfere with the pH-induced conformational change of HA required for fusion. Unfortunately, such antibodies are far less frequently induced by conventional vaccines.
In this work, the hemagglutinin (HA) gene of A/chicken/Yamaguchi/8/2004 (H5N1) was expressed using a recombinant vesicular stomatitis virus lacking the essential glycoprotein G gene. VSV∆G(HA) was propagated on helper cells providing the VSV G protein *in trans*. Infection of non-helper cell resulted in high level expression of HA in its native conformation but not in release of infectious progeny virus. Immunization (i.m.) of chickens with adjuvant-free VSV∆G(HA) induced antibodies with broadly neutralizing activity against even highly divergent H5 strains. PepSpot™ and Western blot analyses revealed that the antibodies bound to epitopes in both the HA_2 stalk region and the HA_1 globular head. This feature is unique for the VSV-based vaccine, as animals which had received either live-attenuated or whole virus inactivated vaccines produced antibodies which predominantly bound to HA_1. Immunized chickens were completely protected against challenge with heterologous highly pathogenic A/whooper swan/Mongolia/3/2005 (H5N1). These findings demonstrate that VSV∆G(HA) represents a potent and safe marker vaccine which also protects against antigen-drifted influenza viruses.

Session 7

Virus Cell Interaction

Chair: Roland Zell

Tuesday, September 4th 2012

Key-note lecture: Can PB1-F2 Be Used as a Molecular Signature of Virulence?
Jon McCullers, Memphis, USA

Fragile X Mental Retardation Protein Stimulates Ribonucleoprotein Assembly of Influenza A Virus
Tao Deng, Beijing, China

TRIM22 Restricts Influenza A Virus Infection
Elisa Vicenzi, Milan, Italy

Adaptive Mutations in NEP Compensate for Defective H5N1 RNA Replication in Human Cells
Peter Reuther, Freiburg, Germany

Fragile X Mental Retardation Protein Stimulates Ribonucleoprotein Assembly of Influenza A Virus

Zhuo Zhou[1], Yang Guo[1], Lili Zhao[1], Jingfeng Wang[1], Xue Jia[1], Qinghua Xue[1], Sheng Cui[1], Qi Jin[1], Jianwei Wang[1], Tao Deng[1]*

[1]MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100730, People's Republic of China

The viral ribonucleoprotein (vRNP) of influenza A virus consists of the viral RNA polymerase complex (PB1, PB2 and PA) bound to a genomic vRNA segment that is coated with multiple copies of nucleoprotein (NP). The vRNPs perform viral RNA transcription and replication in the nucleus, which require various interplays between host factors and the RNP components. We performed a cellular transcriptional profiling-based siRNA screening and identified 40 new host genes which were potentially involved in influenza virus replicaiton. Among these genes, we found that *FMR1* (fragile X mental retardation) gene plays an important role in influenza virus replication. Taking advantage of an RNP reconstitution assay, we showed that *FMR1* encoding protein FMRP is directly involved in viral RNA synthesis machinery. Further biochemical experiments revealed that FMRP stimulates RNP assembly by interacting with viral nucleoprotein (NP) in an RNA-dependent manner. The domain mapping experiments demonstrated that the KH2 domain of FMRP is critical for NP association. Interestingly, a point mutation (I304N) in the KH2 domain of FMRP, which perturbs the structure of KH2 domain and is associated with a severe case of Fragile X syndrome, disrupts FMRP-NP association and the ability of FMRP to stimulate viral RNP assembly. Our data suggest a role of FMRP in influenza virus RNP assembly, and also shed new light on FMRP function.

Key words: influenza virus; ribonucleoprotein assembly; FMRP

TRIM22 restricts Influenza A Virus Infection

A. Di Pietro[1], A. Kajaste-Rudnitski[1], G.Towers[2], A. Oteiza[3], N. Mechti[3], E.Vicenzi[1*]

[1]Vita-Salute San Raffaele University and San Raffaele Scientific Institute, Milan; [2]MRC Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London, London; [3]CNRS UMR 5236, Centre d'Étudesd'AgentsPathogènes et Biotechnologie pour la Santé (CPBS), Montpellier.

Influenza A virus (IAV) infection leads to a burst of type-1 interferon (IFN), which triggers an antiviral response through the induction of several genes includingTRIM22,a member of the tripartite motif family.TRIM22 exerts an antiviral activity against Human Immunodeficiency virus, Hepatitis B virus and encephalomyocarditis virus. The objective of this study was to test whether influenza virus belongs to the targets of TRIM22 anti-viral factor.
To investigate whether TRIM22-induction impacted IAV replication, A549 cell lines were depleted ofTRIM22 by short hairpin RNAiexpressed from a lentiviral vector. Multi-cycle growth curves of A/New Caledonia/20/99 (H1N1) indicated that the production of viral particles was increased by two orders of magnitude in A549 cells in which TRIM22 gene was silenced as compared to non-silencing expressing controls.To test whether over-expression of TRIM22 impaired IAV replication, Madin Darby canine kidney (MDCK) cells were transduced with a retroviral vector encoding either anHA-tagged TRIM22-encoding vector or an empty vector as a control. TRIM22 overexpression decreased the IAV titer by 200 folds 48 h post-infection.
Restriction of IAV by TRIM22 relied on direct interaction with the viral nucleoproteinby inducing its ubiquitinationand degradation ina proteasome-dependent manner.These results demonstrate that the E3 ubiquitin ligase activity of TRIM22 restricts IAV infection by targeting the nucleoprotein.

Key words: Influenza A virus, interferon, restriction factors, TRIM22

Adaptive mutations in NEP compensate for defective H5N1 RNA replication in human cells

Peter Reuther[1, 2*], Linda Brunotte[1], Normann Kilb[1], Benjamin Mänz[1], Martin Schwemmle[1]

[1]Department of Virology, [2]Faculty of Biology University of Freiburg, 79104 Freiburg, Germany

Infection of humans by highly pathogenic avian H5N1 influenza viruses depends on the acquisition of adaptive mutations to allow efficient viral replication in the new host. This can be achieved by mutation of the glutamate at position 627 in PB2 to lysine, which has been associated with increased polymerase activity in human cells. We have recently shown, that adaptive mutations in the viral export protein NEP can compensate for the lack of PB2-627K observed in the majority of human H5N1 isolates, leading to enhanced polymerase activity in mammalian cells. Mechanistic characterization of NEP revealed that this polymerase activating function resides in the C terminus of the protein and strongly depends on the integrity of the last three amino acids. In contrast, the N terminus functions as a negative regulator leading to reduced polymerase activation by NEP. We could further show that the adaptive mutations in NEP increases its polymerase enhancing activity by reducing the inhibitory effect exerted by the N-terminus. Most strikingly, NEP function is mediated by a direct interaction of NEP C terminus with the polymerase subunit PB2, which we could show by co-immunoprecipitation studies and functional analysis. A more detailed analysis of the NEP-PB2 interaction demonstrated that NEP targets a 15 amino acid comprising motif of PB2 in proximity to the Cap-binding domain. Together, our results suggest that NEP is a novel and potent co-factor of avian H5N1 polymerase promoting viral replication by acquiring adaptive mutations and direct interaction with the PB2 subunit.

Key words: H5N1, polymerase, adaptation, pathogenicity, NEP

POSTER PRESENTATIONS

P1

Dengue Virus Coinfection Exacerbates Influenza Replication

Benjamin Bradley[1], Mei-Chun Chen[1], Gena Nichols[1], Somanna Naveen[1], Gregor Manukian, Catherine Arnold[1], Courtney Standlee[1], Jackson Voss[1], Alex LaVanway[1], Kayleigh Voss[1], Courtney Garry[1], Thomas Voss[1*]

[1]Tulane University School of Medicine, New Orleans, LA USA

Dengue fever and Dengue Hemorrhagic fever in humans are the result of infection with Dengue virus, a mosquito-borne member of the *Flaviviridae.* Dengue infection occurs in an estimated 50-100 million infections annually in endemic regions with significant morbidity and mortality. In 2009, the appearance of a novel, swine origin, pandemic (H1N1) influenza A virus in humans led to an observation of increased morbidity and mortality for patients with concurrent Dengue *and* influenza infections. To Examine the interaction of Dengue and flu during co-infection, we performed cell culture and animal experiments in A549 cells and ferrets. In A549, co-infection enhanced influenza virus replication and reduced dengue virus replication compared to singly infected A549 cells. Indirect immune fluorescence for flu or dengue show that, while there is evidence of infection with both viruses in vitro, few cells are dually infected, support and indirect role for influenza-mediated restriction of Dengue infection and dengue-mediated enhancement of influenza replication. Studies in ferrets co-infected with both Dengue and pandemic influenza show alterations in clinical hematology, virus loads, and clinical disease compared to infection with each virus individually. In both models, there was a reduction in influenza-mediated apoptosis during co-infection as measured by TUNEL, Anexin V staining, and by expression of Caspase-3, a key mediator of apoptosis. The delay or abrogation of influenza-mediated apoptosis may be an important correlate of pathogenesis in co-infected humans and may lead to a clearer understanding of the virus-virus interactions that may be important in influenza outbreaks in dengue-endemic regions globally.

Key words: Dengue, Influenza, Ferret, Apoptosis

P2

Avian influenza viruses inhibit the major cellular signaling integrator c-Abl

Eike R. Hrincius[1], Swantje Liedmann[1], Darisuren Anhlan[1], Thorsten Wolff[2], Stephan Ludwig[1] and Christina Ehrhardt[1*]

[1] Institute of Molecular Virology (IMV), Center of Molecular Biology of Inflammation (ZMBE), University of Muenster, Von Esmarch-Str. 56, D-48149 Muenster, Germany
[2] Div. of Influenza/Respiratory Viruses, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany

The non-structural protein 1 (NS1) of influenza A viruses (IAV) encodes several src homology (SH) binding motifs (bm) (one SH2bm, up to two SH3bm), mediating interactions with host cell proteins. Since sequence analysis revealed a different avian (class II SH3bm) versus human (no SH3bm) consensus sequence for the second SH3bm (SH3(II)bm) and based on our former studies, which showed that NS1 binding to CRK adaptor proteins is mediated via this motif, we monitored the regulatory properties of this SH3bm for cellular signaling and the functional consequences in IAV life cycle. We observed that the basal phosphorylation level of CRK was clearly reduced upon infection with different avian IAV harboring an NS1 with an SH3(II)bm in contrast to NS1 of human IAV strains. Reduced activity of the tyrosine kinase c-Abl was identified to be responsible for reduced CRK phosphorylation. Further, direct binding of NS1 to c-Abl was determined. Mutational manipulation of the SH3(II)bm illustrated the necessity of this motif for c-Abl inhibition. Interestingly, Abl kinase inhibition resulted in impaired avian IAV propagation and mutational analysis linked the pronounced inhibition of c-Abl to cytopathic cell alterations upon avian IAV infections. In summary, our data illustrate that NS1 proteins of avian IAV interfere with the kinase activity of c-Abl, a major cellular signaling integrator that controls multiple signaling processes and cell fate regulations apparently including IAV infections.

Key words: influenza A virus, Abl kinase, cellular signal transduction

P3

Broadly protective trivalent recombinant baculoviral vaccine against heterologous influenza H5N1 challenge

M. Prabakaran* & J. Kwang

Animal Health Biotechnology, Temasek Life Sciences Laboratory, National University of Singapore

The rapid evolution of new sub-lineages of H5N1 subtype in poultry population is considered to be the most likely culprit for the next pandemic. Zoonotic H5N1 influenza transmission resulted in 604 confirmed human infections and 357 fatalities (as of 29th May 2012). The availability of safe and broadly protective vaccines for avian influenza H5N1 is high priority in preparedness for influenza pandemic. In this study, hemagglutinin (HA) genes of three different H5N1 strains (A/Vietnam/1203/2004; A/Indonesia/CDC669/2006 & A/Anhui/01/05) were selected based on the epitope mapping and distribution analysis of neutralizing epitopes among H5N1 lineages. The selected HAs were expressed on the baculovirus surface (Trivalent-BacHA) and evaluated cross-protective efficacy against H5N1 influenza in a mouse model. The group of mice vaccinated with trivalent-BacHA vaccine generated a robust immune response evident by hemagglutination inhibition (HAI) titer and serum micro-neutralization titer. Moreover, trivalent-BacHA vaccine effectively neutralized viruses from distinct clades/subclades (0, 1, 2.1, 2.2, 2.3, 4, 7 & 9) of H5N1 strains compared to monovalent-BacHA. Further, trivalent-BacHA vaccine exhibited cross-protection against 5MLD50 of distinct clade (clade 7) of H5N1 strain. The present findings revealed that inclusion of three HAs from divergent H5N1 strains will help prevent infection during prepandemic situation where the eventual pandemic strain is unknown.

Key words: Influenza H5N1, pre-pandemic vaccine, recombinant Baculovirus

P4

Modeling influenza virus replication on a single cell level

T. Frensing*, F. S. Heldt, U. Reichl

Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany

Influenza viruses transcribe and replicate their negative-sense RNA genome inside the nucleus of host cells via three viral RNA species: the genomic vRNA(-), the replicative intermediate cRNA(+) and the viral mRNA(+). In the course of an infection, these RNAs show distinct dynamics suggesting that differential regulation takes place. In order to investigate this regulation in a systematic way, we developed a mathematical model of influenza virus infection at the single cell level. It accounts for key steps of the viral life cycle from virus entry to progeny virion release while focusing in particular on the control of viral transcription and replication. Therefore, two mechanisms are considered in the mathematical framework. At early stages of an infection transcription dominates until newly synthesized viral polymerase complexes and nucleoproteins stabilize the cRNA, which subsequently facilitates the synthesis of genomic vRNAs. Later on, the nuclear export of viral genomes (vRNPs) controls RNA levels. In preparation for their export vRNPs become inactivated and serve no longer as templates for the synthesis of viral mRNA and cRNA. Both mechanisms together allow the model to capture the dynamics of viral RNA levels form a variety of published data sets. Moreover, simulations show an accumulation of viral proteins and RNA toward the end of infection indicating that transport processes or budding may limit virion release. Thus, our mathematical model provides an ideal basis for a systematic and quantitative evaluation of influenza virus replication and its complex regulation.

Key words: mathematical modeling, regulation of RNA synthesis

P5

Single mutations in HA and NP mediate enhanced pathogenicity of 2009 H1N1 pandemic influenza virus in mice

A. Otte* and G. Gabriel

Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

The emergence of the influenza pandemic in 2009 underlines the constant threat to humans caused by influenza A viruses. Although the 2009 H1N1 pandemic (pH1N1) was predominantly mild, many severe cases were reported among young adults without any underlying disease. Viral pathogenicity markers described before were not present in the 2009 pH1N1 influenza virus strains. Therefore, novel determinants of 2009 pH1N1 pathogenicity need to be identified. Recently, we proposed that C57BL/6J mice are a more suitable small animal model to study 2009 pH1N1 pathogenesis than commonly used BALB/c mice. While 2009 pH1N1 pathogenicity markers were masked in BALB/c, they became visible in C57BL/6J mice without the need of prior adaptation (Otte et al. 2011; Otte and Gabriel 2011).

Here, we have assessed the differential virulences of two 2009 pH1N1 clinical isolates in the C57BL/6J mouse model. The low pathogenic HH05 (pH1N1) (MLD_{50}:5.2 p.f.u.) differs from the high pathogenic HH15 (pH1N1) (MLD_{50}:3.5 p.f.u.) by 12 amino acid substitutions. To analyze the viral determinants responsible for enhanced 2009 pH1N1 virulence, we have generated several single-gene reassortant and single point-mutant viruses by reverse genetics. We have identified novel single mutations in the viral nucleoprotein (NP) and in the hemagglutinin (HA) which enhance 2009 pH1N1 virulence in C57BL/6J mice. Currently, we are investigating the molecular mechanisms by which these mutations mediate enhanced 2009 pH1N1 pathogenicity. The results obtained in this study reveal novel 2009 pH1N1 pathogenicity markers and may eventually lead to the development of novel antiviral strategies against pandemic influenza viruses.

Key words: pandemic H1N1, differential pathogenesis

P6

Host serine proteases mediate differential cleavage of HA of different H1N1-PR8 variants

Mahmoud M. Bahgat* and Klaus Schughart

Department of Infection Genetics and University of Veterinary Medicine, Helmholtz Center for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

Recently, we reported the possible implication of multiple serine protease activities in mediating influenza A HA cleavage and infection and showed the capacity of serine protease inhibitors to block virus infection both in cultured lung cells and in mice. Here, we report on differential serine protease activities in embryonated chicken eggs, mouse lungs and cultured mammalian cells in response to infection with different variants of H1N1-PR8 isolates. Also we present evidence on differential cleavage of HA of such PR8 strains in infected embryonated chicken eggs. Studies of HA cleavage in infected cultured mammalian cells and lungs of mice with various susceptibilities to influenza infection are in progress. The impact of these findings on influenza virulence will be discussed.

Key words: Influenza A viruses, PR8 variants, trypsin-like serine proteases, HA cleavage

P7

The influenza virus PB1-F2 protein has interferon-antagonistic activity

S. E. Dudek*, L. Wixler, C. Nordhoff, A. Nordmann, D. Anhlan, V. Wixler, and S. Ludwig

Institute of Molecular Virology (IMV), Centre for Molecular Biology of Inflammation (ZMBE), Westfälische Wilhelms-University, D-48149 Münster, Germany

PB1-F2 is a non-structural protein of influenza viruses encoded by the PB1 gene segment from a +1 open reading frame. It has been shown that PB1-F2 contributes to viral pathogenicity even though the underlying mechanisms are still under debate.
Induction of type I interferon (IFN) is a first line of defence against viral infections. Here we show that influenza A viruses lacking the PB1-F2 protein induced enhanced expression of IFN-β and interferon-stimulated genes (ISGs) in infected epithelial cells demonstrating a type I IFN-antagonistic activity of PB1-F2. On a molecular level PB1-F2 interfered with the RIG-I/MAVS protein complex thereby inhibiting the activation of the downstream transcription factor IRF-3. These findings were also reflected in *in vivo* studies demonstrating that infection with PR8 wild type (wt) virus resulted in higher lung titres and a more severe onset of disease compared to infection with its PB1-F2 deficient counterpart. Accordingly, a much more pronounced infiltration of lungs with immune cells was detected in mice infected with the PB1-F2 wt virus.
In summary, we have identified PB1-F2 as a second type I IFN-antagonistic protein of influenza A viruses that acts via interference with the RIG-I/MAVS complex, thereby contributing to enhanced pathogenicity *in vivo*.

Key words: IFN-antagonist; MAVS; PB1-F2; RIG-I.

P8

Tracking influenza virus fusion – Relation between endosomal pH and stability of the influenza hemagglutinin

C. Mair[1*], T. Meyer[2], K. Schneider[1], T. Korte[1], A. Herrmann[1]

[1]Humboldt University Berlin, Dept. of Biology, Invalidenstr. 42, 10115 Berlin, Germany, [2]Free University Berlin, Institute of Chemistry and Biochemistry, Fabeckstraße 36a, 14195 Berlin, Germany

The fusion initiating conformational change of hemagglutinin (HA) is mediated by a water-driven “spring-loaded mechanism” which requires protonation of the HA1 globular heads in the acidic environment of the endosome.
It has been shown for the HA of influenza virus X-31 that the pH of fusion can be shifted by introducing or deleting salt bridges in this domain. It was hypothesized that the double mutation T212E-N216R leads to the formation of a salt bridge and thus to a shift of the pH of fusion to lower values. However, using experimental approaches and Molecular Dynamics studies, we suggest that the introduction of charges in the vicinity of His 184 modulates its protonation state, thus altering pH dependant fusion activity.
As a proof of concept recombinant virus particles containing wildtype or mutant H3 were produced and fusion kinetics was assessed by fluorescence dequenching. The T212E-N216R mutation has a significant effect on the pH profile of fusion efficiency. Interestingly, Arg at position 216 is naturally present in the highly pathogenic avian influenza HA (H5 HP) whereas the low pathogenic HA (H5 LP) has a Glu at this position. We could show that the exchange of these charges in both, H5 LP and HP, rescues the pH dependence of fusion of HP and LP, respectively, supporting our hypothesis. We further suggest that influenza viruses can adjust their pH of activation by the introduction or exchange of charged residues in the vicinity of other conserved His, thereby regulating pH of fusion and stability.

Key words: recombinant viruses, Histidines, avian HA, fusion pH

P9

Human-like PB2 627K influenza virus polymerase activity is regulated by importin-α1 and -α7

Ben Hudjetz*, Gülsah Gabriel

Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

Influenza A viruses may cross species barriers and transmit to humans with the potential to cause pandemics. Interplay of human- (PB2 627K) and avian-like (PB2 627E) influenza polymerase complexes with unknown host factors have been postulated to play a key role in interspecies transmission. Here, we have identified human importin-α isoforms (α1 and α7) as positive regulators of human- but not avian-like polymerase activity. Human-like polymerase activity correlated with efficient recruitment of α1 and α7 to viral ribonucleoprotein complexes (vRNPs) without affecting subcellular localization. We also observed that human-like influenza virus growth was impaired in α1 and α7 downregulated human lung cells. Mice lacking α7 were less susceptible to human- but not avian-like influenza virus infection. Thus, α1 and α7 are positive regulators of human-like polymerase activity and pathogenicity beyond their role in nuclear transport.

Key words: influenza polymerase activity, importins, influenza host adaptation, PB2 627K

P10

Adaptation of influenza vaccine donor strain in Vero cells results in elevated viral polymerase activity

Ke Xu*, Weibin Hu, Hong Zhang and Bing Sun

Molecular Virus Unit, Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025 China

The current substrate for influenza vaccine production, embryonated chicken eggs, has many inherent drawbacks, so that cell culture-based vaccine production was supposed to be an upgrading way to produce large-scale influenza vaccines. African green monkey kidney cell line (Vero) is widely used in human vaccine manufacture and its safety has been proofed. However, the most commonly used backbone donor virus for influenza vaccine, A/Puerto Rico/8/34 (PR8) virus, is an egg-adapted strain that could not grow efficiently in Vero cells.
In this study, the PR8 virus was adapted to Vero cells by continuous passages. A high-growth strain was gained after 20th passages, which grew 1,000 fold faster than the original PR8 virus without changing the pathogenicity neither in chicken embryos nor in mice. Sequence analysis and growth property assays of the adaptive strain revealed that four mutations in internal viral genes contributed to Vero adaptive property. Recombinant virus harbouring these four mutations showed an increased polymerase activity, and could serve as a backbone donor virus to support the growth of avian H7N1 and H9N2 viruses in Vero cells. Interesting, cellular interactions of viral NP protein in infected cells were observed to be different between the original and adaptive PR8 viruses, suggesting the potential mechanisms for elevated polymerase activity and the adaptation.
Thus, a Vero-adaptive high-yielding PR8 virus was developed by introducing four mutations in viral internal genes which can be used as a donor virus to efficiently produce influenza vaccines in Vero cells.

Key words: influenza vaccine; donor strain; Vero adaptation; polymerase activity

P11

Comparative immunological investigations in mice after infection with mouse-adapted variants of a classical H1N1 Swine Influenzavirus and the pandemic H1N1/2009 Influenzavirus

J. Bogs*, U. Blohm

Institute for Immunology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald – Insel Riems

H1N1 Swine Influenzaviruses (SIV) are endemically distributed in pigs and together with H3N2 viruses the main cause of respiratory diseases in that reservoir host. In 2009 the human population was faced with a new H1N1 strain which also originated from pigs (S-OIV) and caused the first (human) influenza pandemic of the 21st century ('pig flu'). Previous investigations in pigs after infection with representatives of SIV and S-OIV revealed considerable differences in the immune response.

To compare the cellular and humoral immune response in mice, we generated mouse-adapted variants of the classical H1N1 SIV A/Swine/Belzig/2/01 and the S-OIV A/Regensburg/D6/09 by passaging the viruses in the lungs of C57BL/6 mice. Subsequently, we determined differences in genome sequences and mouse virulence of the adapted variants compared to their parental strains. Finally, we infected C57BL/6 mice with the mouse-adapted SIV and S-OIV variants to identify differently regulated factors of the immune system that may as well contribute to the altered immune response in pigs.

We will present the amino acid substitutions and the differences in virulence (LD_{50} and viral load in lungs) observed after mouse-adaptation as well as the results of the immunological investigations in mice.

Key words: Swine Influenzavirus, pandemic Influenzavirus, adaptation to mice, cellular immunity, humoral immunity

P12

Rps6 phosphorylation is required for efficient Influenza infection

C. Carron[1], O. Terrier[1], G. Therizols[2], B. Panthu[3], G. Cartet[1], A. Traversier[1], F. Janaud[1], S. Hacot[2], B. Lina[1], T. Ohlmann[3], S. Ludwig[4], J-J. Diaz[2], M. Rosa-Calatrava[1*]

[1]Equipe VirCell, Laboratoire de Virologie et Pathologie Humaine, VirPath EMR 4610, Université de Lyon, Université Claude Bernard Lyon 1 – Hospices Civils de Lyon, Faculté de médecine RTH Laennec, rue Guillaume Paradin, F-69008, Lyon, France ; [2] Centre de Recherche en Cancérologie de Lyon (CRCL), UMR Inserm U1052 CNRS 5286 UCBL Centre Léon Bérard, 28 rue Laennec, F-69373 Lyon cedex 08, France ; [3] INSERM U758, Lyon F-69364, Ecole Normale Supérieure de Lyon, Université de Lyon, Lyon F-69364, Inserm U851, IFR128 BioSciences Lyon-Gerland, Lyon F-69007 ; [4] Institute of Molecular Virology, Center of Molecular Biology of Inflammation, University of Muenster, Muenster, Germany

Influenza A viruses replicate within the host cellular nucleus. During infection, cellular expression is shut off by different viral mechanisms, as (i) the “cap-snatching” of cellular mRNAs, (ii) the inhibition of their nucleo-cytoplasmic export and (iii) the hijacking of translational machinery. At the same time, infection induces a strong modification of the nucleolar ultrastructure and composition. Unexpectedly, this remodelling is not associated with an inhibition of the ribosome biogenesis, but rather with an increase of rDNA transcription and ribosomal protein synthesis. Furthermore, we have observed that infection induces both alterations of ribosome biogenesis process and several post-translational modifications of ribosomal proteins. Based on these results, we have hypothesized that viruses could also hijack the ribosome biogenesis in support of an effective viral protein translation.

We have focused on a particular ribosomal protein, rps6, whom phosphorylation is up-regulated in purified ribosomes from infected cells. Rps6 phosphorylation is known to be involved in the regulation of translation; however mechanisms carried out are still unclear and functional significance of rps6 phosphorylation seems to be divergent according different cellular types.

In human epithelial cells A549, our results demonstrate that, rps6 is mainly phosphorylated by the kinase S6K1, following the viral activation of the PI3K pathway. Moreover, by using specific S6K1 inhibitor and mouse knockin rps6P -/- cell line, we demonstrate that rps6 phosphorylation is *per se* required for efficient viral protein expression and virus production. These results suggest rps6 phosphorylation could be involved in the regulation of viral mRNA translation during infection.

Key words: Influenza, Rps6 phosphorylation, Pi3K/Akt

P13

Influenza A viruses control the expression of pro-viral human p53 isoforms, p53β and Δ133p53α in human lung epithelial cells

O. Terrier[1*], V. Marcel[2], G. Cartet[1], D.P. Lane[3], B. Lina[1], J.C. Bourdon[2], M. Rosa-Calatrava[1]

[1.] Laboratoire de Virologie et Pathologies Humaines EA4610 VirPath, VirCell Team, Université de Lyon, Lyon, France [2.]Division of Medical Science, Centre for Oncology and Molecular Medicine, University of Dundee, Ninewells Hospital, Dundee, Scotland, UK [3]p53 Laboratory (p53Lab), 8A Biomedical Grove, #06-06, Immunos, Singapore 138648, Singapore

Previous studies have described the role of p53 isoforms, including p53β and Δ133p53α, in the modulation of activity of the full-length p53, which regulates the cell fate outcome. In the context of influenza infection, an interplay between influenza viruses and p53 has been described, p53 being involved in the antiviral response. However, the role of physiological p53 isoforms has never been explored in this context. Here, we demonstrate that p53 isoforms play a role in influenza A virus infection, using silencing and transient expression strategies in human lung epithelial cells. In addition, with the help of a panel of different influenza viruses from different subtypes, we also show that the infection differentially regulate the expression of p53β and Δ133p53α. Altogether, our results highlight the role of p53 isoforms in the viral cycle of influenza A viruses, p53β and Δ133p53α acting as regulators of viral production in a p53-dependent manner. These new data may help to further understand the antiviral activity of p53 and more largely the multiple facets of p53 in influenza-host interactions.

Key words: p53;p53 isoforms; influenza viruses; virus/host interactions

P14

Differential effects of the flavonoids biochanin A and baicalein in H5N1 influenza A virus-infected cells

M. Michaelis[1*], P. Sithisarn[2], M. Schubert-Zsilavecz[3], J. Cinatl jr. [2]

[1]School of Biosciences, University of Kent, Canterbury, UK; [2]Institut für Medizinische Virologie, Klinikum der Goethe-Universität, Frankfurt/Main, Germany; [3]AInstitut für Pharmazeutische Chemie, Goethe-Universität, Frankfurt/Main, Germany

From a panel of 22 flavonoids, we identified six compounds (apigenin, baicalein, biochanin A, kaempferol, luteolin, naringenin) that inhibited influenza A nucleoprotein production in human lung epithelial (A549) cells infected with the highly pathogenic avian influenza virus strain A/Thailand/Kan-1/04 in non-toxic concentrations. Among these flavonoids, biochanin A and baicalein displayed the highest selectivity indices (5.6 and 5.8, respectively) and were chosen for further investigations. Biochanin A and baicalein reduced H5N1 titres. Further, they suppressed the virus-induced caspase 3 cleavage, the nuclear export of viral RNP complexes, and enhanced the effects of the neuraminidase inhibitor zanamivir. Biochanin A and baicalein also inhibited the replication of the H5N1 strain A/Vietnam/1203/04. Time of addition experiments indicated that both compounds interfere with H5N1 replication after the adsorption period. However, further mechanistic investigations revealed clear differences between these two flavonoids. Baicalein but not biochanin A interfered with the viral neuraminidase activity in the used concentrations. In contrast to baicalein, biochanin A affected cellular signalling pathways resulting in reduced virus-induced activation of AKT, ERK 1/2, and NF-kB. Moreover, biochanin inhibited the virus-induced production of IL-6, IL-8, and IP-10 while baicalein inhibited IL-6 and IL-8 production without affecting IP-10 levels. In primary human monocyte-derived macrophages, only baicalein but not biochanin A impaired H5N1 virus replication. Both flavonoids interfered with the H5N1-induced production of IL-6, IP-10, and TNF-alpha but not of IL-8 in macrophages. These findings indicate that closely related flavonoids can exert anti-influenza effects by different molecular mechanisms.

Key words: H5N1, flavonoid, biochanin A, baicalein, antiviral

P15

A novel single-virus infection system reveals that influenza virus preferentially infects resting cells

Ayae Honda[1*],Tadao Sugiura[2], Fumihito Arai[3]

1.Hosei University, Tokyo , Japan, 2. Nara Institute of Science and Technology, Nara, Japan, 3. Nagoya University, Nagoya, Japan

It has been shown that influenza virus attaches sialic acid on the cell surface, enter into cell by endocytosis. At the late endosome, the viral RNP (RNA genome, RNA dependent RNA and NP complex) is released from the viral particle and enter to nucleus. Until now it is still unclear which cell phase influenza virus select for infection. To solve these questions we developed the single virus infection system using optical tweezers at 1064 nm. We found that influenza virus selectively bound to resting cells. This result prompted us to study further analysis. We performed to analyze the sialic acid content, endosomal protein, membrane stiffness and lipid. Sialic acid content and the proteins for endosome formation were greater in resting than dividing cell. We also analyzed the membrane stiffness of resting and dividing cells, and found that the stiffness of dividing cell is greater than resting cell.

Key words: resting, dividing, optical tweezers

P16

Isolation and characterization of H9N2 avian influenza virus from Bangladesh

R. Parvin* and T.W. Vahlenkamp

Institute of Virology, Faculty of Veterinary Medicine, University of Leipzig, Germany

Avian influenza viruses of subtype H9N2 have become widespread in poultry in many countries with clinical relevance in respiratory diseases with multifactorial origin. Besides highly pathogenic H5N1 avian influenza viruses Bangladesh also confirmed the presence of low pathogenic H9N2 in commercial and backyard poultry. Retrospective analysis of a sample received from Bangladesh (A/Ck/BD/VP01/2007) led to the isolation of a H9N2 avian influenza virus which was propagated twice in embryonated chicken eggs. The allantoic fluids were harvested for further characterization. The isolation of the virus was confirmed by real time RT-PCR for influenza type A matrix protein gene. HA titer of the propagated virus was deterimed between 256-512 HA units. Subtyping by RT-PCR using 383bp and 244 bp spanning fragments of the H9 and N2 gene, respectively was performed and full length amplification of HA and M gene of H9N2 virus was done. The RT-PCR products were cleaned, sequenced and a phylogenetic tree was generated with comparisons to other available sequences at the NCBI genbank. The nucleotide blast search of HA and M gene sequences revealed that the Bangladeshi isolate is closely related with 99% homologies to Indian isolates and with homologies between 96 and 98% to other south east Asian isolates of H9N2. Along with other isolates from South Asia and Middle East the Bangladeshi isolate belong to ‘quail/HK/G1/97-like’ sub-lineage of H9N2 viruses.

Key words: Low pathogenic avian influenza, H9N2, Phylogenetic analysis

P17

Role of the amphiphilic helix of M2 and the cytoplasmic tail of HA for virus budding

M. Veit[1*], S. Sieche[1], K. Brett[1], T. Wolff[2], A. Herrmann[3], B. Thaa[1]

[1] Freie Universität Berlin, Fachbereich Veterinärmedizin, Institut für Immunologie, Philippstraße 13, 10115 Berlin, [2] Robert-Koch-Institut, Nordufer 20, 13353 Berlin, [3] Humboldt-Universität zu Berlin, Institut für Biologie, Molekulare Biophysik, Invalidenstraße 42, 10115 Berlin

Influenza virus assembly occurs in the “budozone”, a coalesced raft- domain. HA requires S-acylation at transmembrane and cytoplasmic cysteines for raft-association and its cytoplasmic tail attracts M1 to the budding site. M2 is implicated in scission of virions, probably by wedge-like membrane insertion of an amphiphilic helix in its cytoplasmic tail. S-acylation of the helix and binding to cholesterol are believed to target M2 to the edge of the budding site.

We have shown previously that both S-acylation and cholesterol-binding affect subcellular targeting of M2 and membrane binding of its cytoplasmic tail. To investigate whether these features are relevant for virus replication, we generated recombinant viruses in the WSN background by mutating the acylation and cholesterol-binding sites of M2, either alone or in combination. Surprisingly, all mutant viruses had similar growth kinetics as wildtype in various cell types. The mutations affected the relative incorporation of M2, but not of the other viral proteins into virions. Co-infection experiments revealed that the competitive fitness of the virus was not impeded by any of the mutations. Thus, acylation and cholesterol-binding in M2 are not crucial for replication of influenza virus.

In contrast, recombinant viruses with mutations in the cytoplasmic tail of HA could either not be rescued or had severe growth defects. The reduction in titre of a mutant with a deleted acylation site in HA was aggravated when the acylation site in M2 was replaced in addition. This points to a synergistic role of HA and M2 acylation for virus budding.

Key words: assembly, budding, HA, M2, S-acylation

P18

Prior vaccination with livevirus protects highly susceptible DBA/2J mice from lethal influenza A H1N1 infection

Leonie Dengler*, Esther Wilk, Mathias May, Mahmoud Bahgat, and Klaus Schughart

Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig and University of Veterinary Medicine, Hannover

The mouse represents an important model system to study the host response to influenza A infections and to evaluate new prevention or treatment strategies. We and others reported that the susceptibility to influenza A infections strongly varies among different inbred mouse strains. In particular, DBA/2J mice are highly susceptible to many influenza A subtypes and they exhibit severe symptoms for clinical isolates without even prior virus adaptation to the mouse. However, until now, it has not been if DBA/2J mice are able to mount a protective immune response against influenza A virus upon vaccination.Here, we demonstrate that afterintra-muscular immunization with viable influenza A virus, DBA/2J mice mounted a virus-specific IgG response and were protected against a subsequent lethal challenge. The antibodytitersafter immunization, theprotection from body weight loss and death inimmunized DBA/2J mice wassimilar to the responsesobserved for immunized C57BL/6J mice. In addition, we demonstrated that DBA/2J mice were sensitive to infection with the pandemic H1N1 virus if vaccinated with the Brisbane H1N1 variant, but were protected when vaccinated with the pandeminc H1N1 virus. In conclusion, DBA/2J mice represent a highly valuable mouse model to evaluate protective potentialof vaccine strains against existing and newly emerging human influenza virus strains without the need for prior adaptation of the virus to the mouse.

P19

Pseudotyped Newcastle Disease Virus with Paramyxovirus 8 Surface Glycoproteins and Highly Pathogenic Avian Influenza Virus Hemagglutinin

Steglich C.[1*]; Grund Ch.[2]; Höper D.[2]; Keil G.[1]; Mettenleiter T.C.[1] and Römer-Oberdörfer A.[1]

[1]Institute of Molecular Biology; [2]Institute of Diagnostic Virology; Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, D-17493 Greifswald-Insel Riems, Germany

The capability of Newcastle disease virus (NDV) as a vaccine vector virus which conveys protection against highly pathogenic avian influenza virus (HPAIV) has already been shown. However, a sustained success has not been achieved since most poultry is routinely vaccinated against NDV. Pre-existing NDV antibodies have a negative influence on vector virus replication, resulting in a lower immune response also against a heterologous protein like HPAIV hemagglutinin. The development of a pseudotyped NDV which possesses functional surface glycoproteins not derived from NDV could overcome this problem.
Here, we describe the construction of a pseudotyped vector NDV (PNDV-FHN$_{PMV8}$H5) which carries both fusion protein (F) and hemagglutinin-neuraminidase protein (HN) of avian paramyxovirus type 8 instead of the corresponding NDV proteins. Additionally, a gene expressing HPAIV H5 was inserted between the PMV-8 F and HN genes. The NDV backbone is derived from the lentogenic NDV strain Clone 30 as already used for other NDV/AIV recombinants. After successful virus rescue the resulting PNDV-FHN$_{PMV8}$H5 was further characterized. The expression of all three foreign genes was verified by Western blot and immunofluorescense. Furthermore, replication could be detected to high titers *in vitro* and *in vivo*. Animal experiments indicated an excellent protection against lethal HPAIV infection in specific pathogen free (SPF) chicken.
Thus, a new vector virus was generated which can probably be used as a marker vaccine against HPAIV that follows the DIVA (differentiation infected from vaccinated animals) principle and could be effective also in the face of maternal NDV antibodies.

P20

Molecular and immunological characterisation of influenza A(H1N1)pdm09 viruses

N. Ikonen, M. Strengell, M. Haanpää, T. Ziegler and I. Julkunen*

Virology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare (THL), Helsinki, Finland

Influenza A(H1N1)pdm09 virus was the major influenza A virus type circulating in Finland during 2009/10 and 2010/11 influenza seasons. During the 2011/12 season only sporadic A(H1N1)pdm09 virus identifications were made. The nucleotide sequences of the two surface glycoprotein genes, hemagglutinin and neuraminidase were determined from viruses collected from nearly 250 patients. These viruses represented different geographic regions and time periods of these three seasons. Mega4 software was used for sequence comparisons and for the construction of a phylogenetic tree.

Based on the HA gene sequence, viruses from the 2009/10 pandemic wave clustered in four genetic groups. In the HA1 region, which contains all the major antigenic sites, the viruses differed from each other by 0-6 amino acids. The difference between the Finnish viruses and the vaccine virus, A/California/07/2009 was 2-5 amino acids. Since May 2010 eight genetic groups have been circulating worldwide. Finnish viruses from the 2010/11 and 2011/12 seasons clustered in six of these eight genetic groups with the exception of one virus, which was closely related to the vaccine virus. The six genetic groups were identified in all geographic areas of Finland. Some of the genetic groups were observed only during a short period while other groups were detected throughout the 2010/11 epidemic season. The amino acid difference of the HA1 region between epidemic viruses and the vaccine virus is 4-9. During three epidemic years the maximal evolution rate is 2.2% in the entire HA, and 2.8% in the HA1 domain. There is a clear correlation in the number of amino acid changes with the time of sample collection. Most of the amino acid changes accumulated on the surface of HA molecule and some of them were located in important antigenic sites, which may affect antigenic properties of the virus. Similar genetic groups were found based on the NA sequence, but the evolutionary rate of the NA gene was somewhat lower as compared to that of HA gene.

Although the evolution of influenza A(H1N1)pdm09 viruses has progressed, none of the recently detected amino acid changes has given the virus an advantage that would make it a dominant virus and allow it to escape immunity induced by vaccination or natural infection. With few exceptions, viruses from different genetic groups have not shown any significant changes in antigenicity compared with the vaccine virus. However, specific rat reference sera prepared against viruses representing different genetic groups showed up to 4-fold differences in their ability to recognize different virus groups in the hemagglutination inhibition (HI) assay. Serum samples collected from Pandemrix and seasonal influenza vaccinated people showed a more than 50% decline in antibody levels within one year after vaccination. There was also a very significant negative correlation in vaccine-induced immunity in relation to age. Therefore, yearly influenza vaccinations of medical risk groups are warranted.

Key words: pandemic influenza, evolution, immunity

P21

Proteolytic cleavage of influenza A H1N1 hemagglutinin is strongly impaired in *Tmprss2* deficient mice

Bastian Hatesuer[1*], Stephanie Bertram[2], Mahmoud Bahgat Riad[1], Nora Mehnert[1], Stefan Pöhlmann[2], and Klaus Schughart[1]

[a]Department of Infection Genetics, Helmholtz Centre for Infection Research and University of Veterinary Medicine Hannover, 38124 Braunschweig, Germany
[b]Department of Infection Biology, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany

The hemagglutinin (HA) protein plays a critical role in the pathogenesis of influenza. The processing of HA protein during the maturation process of the virus requires proteases of the host and the susceptibility of the HA cleavage site to the host protease determines the virulence of the virus. Low pathogenic viruses, like the human H1N1, H2N2 and H3N2 variants, contain a monobasic cleavage site and are activated by proteases expressed in lung and gastrointestinal tract. In contrast, the cleavage sites of highly pathogenic viruses (subtypes H5, H7) contain multibasic amino acids that are recognized by ubiquitously expressed proteases. It was shown in cell culture experiments that different type II transmembrane serine proteases allow HA cleavage.
Here, we show that deletion of a single HA-activating protease gene, *Tmprss2*, completely protects mice from weight loss and death upon infection with H1N1 influenza viruses. In contrast, deletion of the *Tmprss2* gene in mice had no negative impact on spreading and pathogenicity in the lung of H7N7 influenza virus which bears a multi-basic cleavage site. These results identify TMPRSS2 as a host factor essential for spreading of mammalian influenza viruses in the lung and as a novel target for antiviral intervention in humans.

Key words: H1N1, mouse, knockout, proteases

P22

Studies on the inhibitory activity of influenza virus-specific neuraminidase inhibitors (NAI) against *Pseudomonas aeruginosa*

C. Leitzinger[1*], M. Richter[1], A. Sobke[2], W. Pfister[2], M. Schmidtke[1]

[1] Jena University Hospital, Department of Virology and Antiviral Therapy, Hans-Knoell-Straße 2, 07745 Jena, Germany
[2] Jena University Hospital, Department of Medical Microbiology, Erlanger Allee 101, 07747 Jena, Germany

Pseudomonas aeruginosa (*P.a.*) is an important agent causing secondary infections e.g. pneumonia and sepsis in influenza patients. Like influenza viruses these bacteria are known to possess neuraminidases (NA) – enzymes which are classified as virulence factors supporting biofilm production and adherence. The active site of *P.a.* NA has been shown to be structurally similar to the NA of influenza viruses. This raises the question whether the NA of *P.a.* could represent a target for influenza virus-specific NAI.
Here, the presence and expression of NA in PA01 was confirmed by PCR and sequencing, Southern blot and RT-PCR. In addition, the gene for NA was detected by PCR in 9 of 32 *P.a.* isolates from infected/colonized patients.
Broth microdilution technique was applied to exclude a growth inhibitory effect of oseltamivir and zanamivir on *P.a.* at a concentration of up to 1 mM. In contrast to previous reports, no significant effect of oseltamivir and zanamivir on biofilm formation of PA01 (NA positive) or the blood culture isolate Bk6695-10 (NA negative) was detected in test systems using either microtiter plates or peg lids. In contrast, nitroxoline significantly reduced the *P.a.*-produced biofilm at a concentration of 0.17 mM. Further studies are ongoing to evaluate the inhibitory activity of novel NAI, the direct effect of NAI on the bacterial enzyme, and adsorption to host cells.

Key words: Pseudomonas aeruginosa, neuraminidase, neuraminidase inhibitors, bacterial susceptibility, influenza

P23

Evolution of German swine influenza A viruses - a progress report

J.Lange[1*], A.Philipps[1], A.Krumbholz[1,2], R.Dürrwald[3], R.Zell[1]

[1]Dept. of Virology and Antiviral Therapy, Jena University Hospital; [2]Institute for Medical Microbiology and Virology, University of Kiel, Schleswig-Holstein; [3]IDT Biologika GmbH, Dessau-Rosslau

Our aim is the genetic characterization of German influenza A virus isolates that were collected during the ongoing swine influenza surveillance of IDT Biologika since 2003. Together with human and avian viruses of several sources more than 300 porcine virus strains have been sequenced with conventional and next generation sequencing technology. Phylogenetic analyses of all eight segments reveal the evolution of the European swine influenza virus lineages since their emergence. Beside the prevalent avian-like H1N1 and the human-like H1N2 and H3N2 lineages, several unusual H1N2, H3N2 and H3N1 reassortants were observed. After emergence of the pandemic H1N1 (2009) virus, an increased number of zoonotic human-to-swine and swine-to-swine transmissions was detected. A H1N2 reassortant comprising segments of the European swine H3N2 lineage and the pandemic H1N1 virus has established a stable infection chain and was frequently isolated since 2010.

Key words: swine influenza surveillance, phylogenetic analysis

P24

Introduction of adaptive mutations in the PB2-subunit of A/Hamburg/05/2009 (H1N1) increase the *in vitro* polymerase activity and *in vivo* pathogenicity in the C57BL/6 mouse model

Folker Schwalm[1*], Anna Otte[2], Gülsah Gabriel[2], Hans-Dieter Klenk[1]

[1]Institute for Virology, Philipps-University, Marburg, Germany; [2]Heinrich-Pette-Institute for Experimental Virology and Immunology, Hamburg, Germany

The adaption of an avian influenza virus to a mammalian host usually involves evolution of the viral polymerase. Over the years, several critical positions for adaption of the polymerase have been identified. In the 2009 pandemic H1N1 virus these critical positions in the PB2 subunit still retained the avian signatures. We therefore investigated if the introduction of adaptive mutations in these positions can alter the pathogenicity of the virus in mammals. We also wanted to further characterize the adaptive potential of the amino acid configuration at position 714 in the PB2-subunit (I or R). Furthermore we studied the effect of amino acid exchanges in position 590/591 which were reported to compensate for the "missing" adaptive exchange in PB2 position 627.

After introducing the mutations E627K, D701N and S714I / S714R in the PB2-subunit of A/Hamburg/05/2009 (H1N1) we analyzed the *in vitro* polymerase activity in mammalian (HEK293T) cells. Corresponding double and triple mutants were also generated and additionally the compensatory mutations in PB2 position 590/591 were removed. Recombinant viruses were rescued and tested for pathogenicity in C57BL/6-mice.

All adaptive mutations showed an elevated polymerase activity *in vitro* compared to the wildtype. While the single substitutions did not alter the pathogenicity in the mouse, there was a significant increase detectable after infection with some of the double / triple mutant viruses. Our observations show that adaptive mutations may further increase the pathogenicity of the pandemic H1N1 virus. Thus, it appears that the virus can further adapt to mammals and that its pathogenic potential has not been exhausted yet.

Key words: H1N1, polymerase, adaptive mutations, pathogenicity

P25

Modulation of the Type I Interferon System by β-Catenin upon Influenza A Virus Infection

A. Hillesheim*, C. Nordhoff, Y. Börgeling, S. Ludwig, V. Wixler

Institute of Molecular Virology (IMV), Centre of Molecular Biology of Inflammation (ZMBE), Muenster, Germany

When cells are infected by viruses, the accumulating viral RNA is recognized by pattern recognition receptors of the host cells leading to activation of several signalling cascades that participate in induction of interferon (IFN) type I genes. While the IFN type I system embodies the predominant part of the innate immune response in influenza A virus infected cells, IFN molecules themselves do not have antiviral potential but activate the JAK/STAT signalling pathway inducing thus the expression of antiviral acting proteins, like MxA, OAS-1 or PKR.
The mammalian homologue of the *drosophila* armadillo protein β-catenin inhere a dual role within the cell. On the one hand it serves as an adapter molecule at the cell membrane linking cell-cell contacts to microfilaments. On the other hand this multifunctional protein forms a bipartite transcription factor with LEF/TCF, when Wnt signalling cascade is activated, regulating gene expression and controlling thus different cellular functions such as cell cycle progression and differentiation.
Here we assign an anti-influenza activity to β-catenin showing that the bipartite transcription factor formed by β-catenin and LEF/TCF enhances the transcription of the IFN-β gene. Being overexpressed in lung epithelial cells β-catenin and LEF/TCF amplify together with CBP/p300 the IRF3-dependent transcription of the IFN-β enhanceosome. Simultaneously the bipartite transcription factor regulates the expression of interferon stimulated genes, but independent to IFN-induced phosphorylation of STAT1 proteins. Consistent with the enhanced activation of the IFN-system and interferon stimulated genes influenza A viruses replicate less efficient in cells overexpressing a degradation resistant mutant of β-catenin.

Key words: type I interferon, β-catenin, influenza A virus

P26

Critical role of segment-specific genome packaging signals in genetic reassortment of influenza A viruses *in vitro.*

M. Yver[1*], B. Essere[1], C. Gavazzi[2], C. Isel[2], O. Terrier[1], E. Fournier[2], T. Julien[1,3], B. Lina[1], M. Rosa-Calatrava[1], R. Marquet[2] ,V. Moules[1,3]

[1]Virologie et Pathologie Humaine, Université Lyon 1, EA4610, Faculté de Médecine RTH Laennec, 69372 Lyon, France ; [2]Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084 Strasbourg, France ; [3]VirNext, Faculté de Médecine RTH Laennec, 69372 Lyon, France

The fragmented nature of the influenza genome allows the exchange of gene segments when two or more influenza viruses infect the same cell. Although genetic reassortment is not random, little is known about the rules underlying this process. Here, we studied genetic reassortment between the human H3N2 (MO) and the avian H5N2 (EN) viruses and found that only a limited subset of all the possible genotypes containing the HA_{EN} gene were produced. Importantly, the wild type HA_{EN} gene never entered the MO genetic background alone but was always accompanied by the PA_{EN} and M_{EN} fragments. Comparing the HA and NA segments, we observed that their capacity to be exchanged during genetic reassortment correlated with the nucleotide sequence identity of these segments between the MO and EN viruses. Furthermore, the use of chimerical HA_{EN} vRNA than contain packaging sequences of the HA_{MO} gene indicated that packaging signals are important for genetic reassortment. We investigated the potential role of specific vRNA/vRNA interactions in genetic reassortment using an in vitro approach. In keeping with the results obtained by this technique, we showed that forcing the incorporation of the M_{EN} segment or a chimerical M_{MO} gene containing five silent point mutations into the 3' packaging region into the MO genetic background was sufficient to drive co-incorporation of the HA_{EN} segment. Altogether, our results indicate that incompatibility between the vRNA packaging signals contributes to limit genetic reassortment in vitro, most likely by perturbing intermolecular vRNA interactions.

Key words: influenza, genetic reassortment, RNA packaging

P27

Cross-Protection between human and European swine H3N2 influenza viruses

Yu Qiu*, Karen van der Meulen, Kristien Van Reeth

Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

H3N2 influenza viruses circulating in humans and European pigs originate from the pandemic A/Hong Kong/68 virus. Because of slower antigenic drift in swine than in humans, the current European H3N2 swine influenza viruses still show some degree of serologic cross-reactivity with human viruses from the 1970s-80s, but not with human viruses isolated after 1990. Using the pig model of influenza, we examined the effect of prior infection with an old (A/Victoria/3/75) or more recent (A/Wisconsin/67/05) human H3N2 virus on protection against challenge with a contemporary swine H3N2 virus (sw/Gent/172/08). Homologous challenge control and previously uninfected challenge control groups were also included. Pigs of the latter group had high titers of the challenge virus in the entire respiratory tract at 3 days post-challenge and nasal virus excretion for 5-6 days. Prior infection with sw/Gent/172/08 or A/Victoria/3/75 offered complete virological protection against challenge. Pigs previously infected with A/Wisconsin/67/05 had similar virus titers in the respiratory tract as the previoiusly uninfected challenge controls, but the mean duration of nasal virus excretion was 1.2 days shorter. These pigs did not have cross-reactive neutralizing antibodies against sw/Gent/172/08 at the moment of challenge, unlike the homologous challenge control and A/Victoria/3/75-immune pigs. Still, antibody titers at 5 days post-challenge were significantly higher in A/Wisconsin/67/05-immune pigs than in the previoiusly uninfected challenge controls. Our data indicate that especially the younger population may be immunologically naïve to European swine H3N2 virus.

Key words: Cross-protection, H3N2 influenza viruses, pigs

P28

Ribavirin but not oseltamivir should be used as control compound in efficacy studies of new anti-influenzaviral compounds against pandemic H1N1 influenza virus (pH1N1) in mice

N. Seidel[1*], H. Braun[1], M. Richter[1], A. Krumbholz[1,2], M. Schmidtke[1]

[1]Jena University Hospital, Department of Virology and Antiviral Therapy, Jena, Germany, [2]University Medical Center Schleswig-Holstein, Department for Infection Medicine, Kiel, Germany

In general, pH1N1 are susceptible against neuraminidase inhibitors (NAI). However, oseltamivir-resistant variants have been described. Moreover, all pH1N1 viruses are naturally resistant against M2-ion channel inhibitors. This emphasizes the search for new anti-influenza drugs.
The aim of the present study was to establish a mouse model based on a NAI-susceptible pH1N1-strain for antiviral studies. Oseltamivir and/or ribavirin were used as control compounds.
According to our previous investigations oseltamivir treatment with 10 mg/kg/d, twice daily, for 5 days has no therapeutic effect on lethal (10^6 $TCID_{50}$) and sublethal (10^5 $TCID_{50}$) infection of female BALB/c mice with A/Jena/5258/09.
In the present study, lethally infected mice were treated (i) with a 10-fold higher dose of oseltamivir, (ii) the administration of this high oseltamivir dose was extended to 7 days, and (iii) ribavirin-treated mice (100 mg/kg/d, twice daily, 5d) were included as additional control group. Neither high oseltamivir dosage nor prolonged treatment revealed a significant effect on survival, changes in body weight, general condition, lung viral titer on d4 p.i., and lung weight as well as histopathology on d4 and 21 p.i. In contrast, ribavirin treatment had a significant impact on most of these parameters. Compared to placebo and oseltamivir-treated mice lung viral titer of ribavirin-treated animals was significantly lower.
Thus, oseltamivir might not be a suitable control compound for testing of new antiviral compounds against pH1N1 in mice and ribavirin represents a good alternative.

Key words: pandemic H1N1, mice, antiviral compounds, oseltamivir, ribavirin

P29

Investigation of the efficacy of Tamiflu® against avH1N1 and huH1N2 swine influenza viruses in pigs

R. Dürrwald[1*], M. Schlegel[1], K. Bauer[2], M. Schmidtke[2]

[1]IDT Biologika GmbH, Dessau-Roßlau; [2]Jena University Hospital, Department of Virology and Antiviral Therapy, Jena

The protective effect of homologous and heterologous vaccination was compared with that of oseltamivir treatment against two Eurasian swine influenza A viruses in their natural host. Vaccinated and Tamiflu®-treated pigs were infected by aerosol nebulisation with high doses of influenza A viruses sw/Bakum/Potsdam/15/1981 (avH1N1) and sw/Bakum/1832/2000 (huH1N2) in parallel trials. Each trail comprised one vaccinated group, one Tamiflu®-treated group as well as one unvaccinated and untreated control group.
Both viruses caused infection and prominent clinical symptoms reflected by fever and dyspnoea in pigs. This allowed the comparative investigation of clinical parameters (dyspnoea, rectal temperatures), lung parameters (viral lung load, macroscopic and microscopic lung lesions) and virus excretion (in nasal swabs).
Tamiflu®-treatment was able to prevent clinical disease and to reduce virus shedding and macroscopic lung lesions but did not influence viral lung load and microscopic lung lesions. Homologous vaccination provided optimal protection against clinical symptoms, lung pathology, and virus replication in the lung. Moreover, a significant reduction of virus shedding was observed. Heterologous vaccination did not prevent virus replication in the lung and macroscopic lung lesions but protected against clinical symptoms, microscopic lung lesions and high virus shedding. All three preventive measures protected against clinical disease and reduced virus shedding but differed regarding the lung parameters.

Key words: oseltamivir, swine influenza, efficacy

P30

Matriptase activates the hemagglutinin of H9N2 influenza A viruses from Asia

Joanna Baron[1], Carolin Tarnow[1], Deborah M. Nüssle[1], Daniela Meyer[2], Torsten Steinmetzer[2], Wolfgang Garten[1], Hans-Dieter Klenk[1], Eva Böttcher-Friebertshäuser[1]*

[1]Institute of Virology, Philipps University, Marburg, Germany; [2]Institute of Pharmaceutical Chemistry, Philipps University, Marburg, Germany

Influenza A viruses of the subtype H9N2 circulate worldwide and have become highly prevalent in poultry in many Asian countries. Moreover, they are occasionally transmitted to humans and pigs, raising concern about their pandemic potential. Influenza virus infectivity requires proteolytic cleavage of the surface glycoprotein hemagglutinin (HA) by host cell proteases. Most influenza viruses are activated at a single arginine (R) by trypsin-like proteases present in a limited number of tissues such as the respiratory or intestinal tract. We identified the type II transmembrane proteases HAT and TMPRSS2 as relevant proteases in the human airways. In contrast, highly pathogenic avian subtypes H5 and H7 are cleaved at the multibasic motif R-X-R/K-R by ubiquitous proteases furin or PC5/6, supporting systemic infection. H9N2 viruses vary remarkably in the amino acid sequence at the cleavage site and many isolates from Asia and the Middle East possess the unusual di- or tribasic motifs R-S-S/R-R, but are not cleaved by furin. Here, we investigated proteolytic activation of different H9N2 viruses containing monobasic (R) or R-S-S/R-R cleavage sites. All H9N2 viruses were activated by HAT and TMPRSS2. Interestingly, H9N2 isolates with R-S-S-R or R-S-R-R cleavage site were also activated by matriptase. The type II transmembrane protease matriptase is expressed in epithelial cells of most tissues. Our data indicate that cleavage of HA by matriptase supports proteolytic activation of H9N2 viruses in a wide range of tissues and may affect organ tropism, viral spread and pathogenicity.

Key words: H9N2 influenza A virus, hemagglutinin, host cell proteases, matriptase

P31

Influenza virus binds its host cell using multiple dynamic interactions revealed by Single Virus Force Spectroscopy and Force Probe MD

C. Sieben[1*], C. Kappel[2], R. Zhu[3], A. Wozniak[4], C. Rankl[5], P. Hinterdorfer[3], H. Grubmüller[2], A. Herrmann[1]

[1]Department of Biology, Molecular Biophysics, Humboldt University Berlin, Berlin, Germany [2]Department of Theoretical and Computational Biophysics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany [3]JPK Instruments AG, Berlin, Germany [4]Institute for Biophysics, Johannes Kepler Universität Linz, Linz, Austria [5]Agilent Technologies Austria, Linz, Austria

Influenza virus belongs to the group of enveloped viruses. The major spike protein of the viral membrane hemagglutinin (HA) binds sialic acid (SA) residues of glycoproteins and -lipids on the plasma membrane of the host cell. This represents the first step of infection and requires multiple simultaneous interactions since the affinity between a single HA-SA pair is very low.
Influenza virus adhesion to living cells was probed by means of dynamic force spectroscopy and force probe molecular dynamics (MD) simulation. We applied three independent approaches to measure the unbinding force between influenza virus and a host cell membrane. Using optical tweezers and AFM based single molecule force spectroscopy we were able to characterize the binding energy on the single molecule level. Unbinding events where analyzed and revealed a multimodal rupture force distribution which suggests sequential binding of multiple receptors. We determined the interacting force between hemagglutinin and its receptor sialic acid to be ~10 pN. Furthermore, we used molecular dynamics simulation to gain detailed information about the binding architecture and the sequence of the unbinding process. We were able to characterize virus specificity on the living cell surface and could verify these results by fluorescence based binding assays.
The combination of experimental and simulated force spectroscopy covers a very large force regime and provides information that could not be obtained with either one or the other method.

Key words: influenza virus, hemagglutinin, cell adhesion, force spectroscopy

P32

Protective efficacy and immunogenicity of a combinatory DNA vaccine against Influenza A Virus and Respiratory Synctial Virus

Viktoria Stab[1], Sandra Nitsche[1], Thomas Niezold[1], Michael Storcksdieck genannt Bonsmann[1], Andrea Thomas[1], Bettina Tippler[1], Christina Ehrhardt[2], Klaus Überla[1], Thomas Grunwald[1] and Matthias Tenbusch[1*,]

[1]Department of Molecular and Medical Virology, Ruhr-Universität Bochum, Germany, [2]Institute of Molecular Virology, ZMBE, Muenster

The Respiratory Syncytial Virus (RSV) and Influenza A Virus (IAV), are both two major causative agents of severe respiratory tract infections in humans leading to hospitalization and thousands of deaths each year. In this study, we evaluated the immunogenicity and efficacy of a combinatory DNA vaccine in comparison to the single component vaccines against both diseases in a mouse model. Intramuscular electroporations with plasmids expressing the hemagglutinin of IAV and the F-protein of RSV induced strong humoral immune responses even if low doses were used. In consequence, high neutralizing antibody titers were detected, which conferred protection against a lethal challenge with IAV. Furthermore, the viral loads in the lungs after a RSV infection could be dramatically reduced in vaccinated mice. Concurrently, substantial amounts of antigen-specific, polyfunctional CD8 T-cells were measured after vaccination with a higher dose of the plasmids. Interestingly, contrary to the antibody responses, where the combinatory DNA vaccine performed equally well as the single component vaccine, the cellular response to the hemagglutinin was significantly reduced in the presence of the RSV-F encoding plasmid. Although these results indicate a suppressive effect of the F-protein the protective efficacy of the combinatory vaccine was comparable to the efficacy of both single-component vaccines. In conclusion, our novel combinatory vaccine against RSV and IAV will have great implications on the rate of severe respiratory tract infections and could further reduce the number of necessary vaccinations.

Key words: DNA vaccine, RSV, Influenza A

P33

Characterization of egg-culture derived, neuraminidase-negative variants of highly pathogenic avian influenza virus H5N1

Donata Kalthoff*, Susanne Röhrs, Dirk Höper, Jürgen Stech, Bernd Hoffmann, Martin Beer
Friedrich-Loeffler-Institut, Greifswald-Insel Riems;

Influenza viruses typically escape from a certain immunogenic pressure by antigenic drift and antigenic shift. To investigate this phenomenon, highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 was forced to replicate under the immunogenic pressure of a neutralizing, polyclonal, chicken-derived serum. After 50 egg-passages, the resulting escape mutants ("EscEgg50A", "EscEgg50B" and "EscEgg50C") revealed major changes in the neuraminidase-encoding segment. Extensive deletions and rearrangements were detected in the new segment 6. Nevertheless, the cleavage site of the hemagglutinin of every "EscEgg50" was still polybasic. Interestingly, the new segments 6 differ between the escape mutants and resulted from complex sequence shuffling and insertion of very short sequence fragments from other segments. Characterization of the escape variants confirmed the loss of neuraminidase activity. By using reverse genetics a recombinant virus consisting of the HPAIV H5N1 backbone and one of the novel segment 6 variants could be generated, while attempts to generate a virus without segment 6 failed. Comparative in vivo characterization defined all escape variants as low-pathogenic influenza viruses, while the control virus was still highly pathogenic after intravenous or oronasal application.
The described virus mutant is to our knowledge the first "neuraminidase-negative" influenza virus generated by immune-escape without external neuraminidase supplementation. In addition, the repeated occurrence of neuraminidase deletions suggests a clear advantage that has to be further determined.

Key words: HPAIV H5N1, neuraminidase

P34

No Apparent Constraints for Novel H3 HA Reassortants with Pandemic Potential

A. Kreibich[1], O. Stech[1], J. Hundt[1, 2], T. C. Mettenleiter[1], and J. Stech[1*]

[1]Friedrich-Loeffler-Institut, Institute of Molecular Biology, Greifswald-Insel Riems, Germany; [2]present address: VIDO-International Vaccine Centre, 120 Veterinary Rd, Saskatoon, SK S7N 5E3, Canada

Reassortment of influenza A virus genes enables antigenic shift resulting in the emergence of pandemic viruses with novel hemagglutinins (HA) acquired from avian or porcine strains. With two H3 avian strains as HA donors, we performed double-infections with a human strain and studied gene compositions and growth of HA reassortants in mammalian cells. To enforce selection for the avian virus HA, we generated a strictly elastase-dependent HA cleavage site mutant from A/Hong Kong/1/68 (H3N2) (Hk68-Ela). This mutant was co-infected with A/Duck/Ukraine/1/63 (H3N8) (DkUkr63) or the more recent A/Mallard/Germany/Wv64-67/05 (H3N2) (MallGer05) in the absence of elastase but presence of trypsin. Both avian strains formed HA reassortants with several gene constellations. Among 21 plaques analyzed from each assay, we found four HA reassortants with DkUkr63 and six with MallGer05 which replicated in A549 cells comparable with the parental human virus. This finding demonstrates that contemporary avian strains may still provide their HA to create a novel potentially pandemic virus. Furthermore, the lower growth of DkUkr63 compared to MallGer05 and Hk68 indicates that lower replication efficiency of the avian virus in a mammalian host may not constrain the emergence of viable HA reassortants. Since reassortment of the HA gene of avian low-pathogenic strains with human viruses is not restricted, adaptation of the HA gene in particular to adequate receptor specificity remains the decisive step for emergence of novel HA reassortants with pandemic potential.

Key words: reassortment, LPAIV, antigenic drift, pandemic strains

P35

Genotypical and phenotypical analysis of two influenza A PR8 variants reveal type I IFN antagonistic properties of influenza polymerase proteins

Swantje Liedmann[1*], Eike-Roman Hrincius[1], Stephan Ludwig[1], Christina Ehrhardt[1]

[1]Institute of Molecular Virology (IMV), Center of Molecular Biology of Inflammation (ZMBE), University of Muenster, Von Esmarch-Str. 56, D-48149 Muenster, Germany

RNA viruses such as influenza A viruses (IAV) have a limited coding capacity due to their small genomes. Therefore, during viral evolution viral proteins with multiple functions have been developed. Here, we did a genotypical and phenotypical comparison of two influenza A virus Puerto Rico/8/34 (PR8) variants to deepen the knwoledge of varied fuctions of different IAV proteins. Overall, we identified 16 amino acid differences in all viral proteins except PB1-F2, M1 and M2. By mutation of these amino acids we were able to dramatically reduce virulence *in vivo* in two different mice strains (Balb/c, C57Bl/6). Interestingly, pathogenicity of both variants is almost similar in IFNAR-/- mice, suggesting virulence regulating properties of the identified amino acids on the level of type I interferon (IFN).
Eight of the identified amino acid variations are located within the polymerase complex and NP, which have well-characterized functions in replication and transcription of vRNA, but also seem to play a crucial role in the inhibition of the innate immune response of host cells. Mutation of these eight amino acids was sufficient for virulence reduction *in vivo*. In addition, our data show clear differences in the induction of the type I IFN response in human lung epithelium, but no differences in the accumulation of viral RNA, suggesting an interplay of the viral polymerase complex and virulence regulation on the level of type I IFN inhibition.
In summary, our data revealed type I IFN antagonistic properties of the polymerase complex as a novel function of these proteins.

Key words: influenza A virus, virulence, type I interferon, polymerase proteins

P36

Influenza A virus entry into cells lacking sialylated N-glycans

Erik de Vries*, Robert P. de Vries, Marleen J. Wienholts, Chantal E. Floris, Marie-Shofie Jacobs, Angelique van den Heuvel, Peter J. M. Rottier and Cornelis A. M de Haan

Virology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Influenza A virus (IAV) enters host cells after attachment of its hemagglutinin (HA) to surface-exposed sialic acids (SIA). Sialylated N-linked glycans have been reported to be essential for IAV entry [Chu and Whittaker (2004) Influenza virus entry and infection require host cell N-linked glycoprotein. *Proc Natl Acad Sci U S* 102:18153-8], thereby implicating the requirement for proteinaceous receptors in IAV entry. Here we show, using different N-acetylglucosaminyl transferase 1 (GnT1)-deficient cells, that N-linked sialosides can mediate but are not required for entry of IAV. Entry into GnT1-deficient cells was fully dependent on sialic acids. Although macropinocytic entry appeared to be affected by the absence of sialylated N-glycans, dynamin-dependent entry was not affected at all. However, binding of HA to GnT1-deficient cells and subsequent entry of IAV were reduced by the presence of serum, which could be reversed by back-transfection of a GnT1-encoding plasmid. The inhibitory effect of serum was much increased by inhibition of the viral receptor destroying enzyme neuraminidase (NA). The results indicate that decoy receptors on soluble serum factors compete with cell surface receptors for binding to HA in the absence of sialylated N-glycans at the cell surface. This competition is particularly disturbed by the additional presence of NA inhibitors, resulting in severely reduced IAV entry. Our results indicate that the balance between HA and NA is not only important for virion release, but also for entry into cells.

Key words: N-linked glycan; Entry; Neuraminidase; GnT1-deficient cell lines.

P37

Comparative analysis of some biological properties of influenza A(H1N1)pdm09 virus strains isolated in Russia in 2009 and 2011

Prokopeva E.A.[1,2,*], Sayfutdinova S.G.[1,2],Ilyicheva T.N.[1,2], Shestopalova L.V.[2], Shestopalov A.M.[1,2]

[1] State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk region, 630559, Russia
[2] Novosibirsk State University, Pirogov St., 2, Novosibirsk, 630090, Russia

The first pandemic of the 21st century began in March of 2009 and was caused by new influenza A(H1N1)pdm09 virus strain of swine origin. During the pandemic influenza A(H1N1)pdm09 virus was registered in more than 214 countries and caused more than 18000 deaths worldwide. Studies carried out in Russia revealed that during the 2009– 2010 season influenza had monoetiological nature that is typically in the pandemic period. Pandemic influenza viruses isolated in Russia from May 2009 to April 2010 were highly homologous to each other and to isolates from abroad, including reference strain A/California/04/2009(H1N1)pdm09. On August 10, 2010 WHO declared cancellation of the 6th threat level for the world and the beginning of the post-pandemic period when influenza A(H1N1)pdm09 virus circulation continued after changing into seasonal infection.
This study objectives were to compare biological characteristics of influenza A(H1N1)pdm09 virus strains having circulated in the pandemic of 2009 and in the post-pandemic period in Russian Asia.
It was shown that pandemic influenza A(H1N1)pdm09 virus strain isolated in the pandemic period is more virulent and able to cause more severe pathological processes in lung and cerebral tissue of experimentally infected mice compared to the strain A(H1N1)pdm09 isolated in the post-pandemic period. It is probably associated with mutations in genes encoding surface proteins that occurred at the time of influenza A(H1N1)pdm09 virus circulation from 2009 to 2011 and that resulted in emergence of new genetic variants of virus with lower virulence.

Key words: A(H1N1)pdm09;virologic, phylogenetic and morphological analysis.

P38

Bcl-2 proteins are central components of host immunity

L. Kakkola, O. Denisova, D. Kainov*

Institute for Molecular Medicine Finland, FIMM

Here we show that cellular Bcl-2 proteins are essential components of host immunity. They are involved in recognition of incoming viruses and activation of host immune responses. The detailed mechanism of this process will be presented.

Key words: immunity, inflammation, infection, apoptosis

P39

MAPKAPK 3 inhibits IFN gamma secretion after flu infection and cytokine stimulation

K. Koether1[1*], C. Nordhoff[1], M. Gaestel[2], J. Bream[3], V. Wixler[1], S. Ludwig[1]

[1]Institute of Molecular Virology (IMV), Centre of Molecular Biology of Inflammation (ZMBE) Westfälische-Wilhelms-University, Von Esmarch-Str. 56, D-48149 Münster, Germany; [2]Hannover Medical School, Institute of Biochemistry, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany; [3]W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205-1901, USA.

The replication of Influenza A virus is strongly dependent on the host cell and in part exploits the cellular signaling machinery for its own purpose. One of the signaling pathways that is activated by virus infection is the p38 MAPK pathway. While this pathway is activated by the cell as part of the antiviral response e.g. to stimulate cytokine expression, it also exhibits virus-supportive activity, e.g. via activation of the p38 downstream substrates MAPKAP kinases 2 (MK2) and 3 (MK3). *In vitro* studies showed that absence of MK2 or MK3 led to a strong reduction of viral titers, most likely due to the lack of PKR inactivation. In *in vivo* studies we were able to confirm the Influenza A virus supportive function of MK3. Mice deficient for MK3 showed a reduction in viral titer and an enhanced survival compared to wt mice. Furthermore, comparative analysis of the immune status of infected mice showed that the MK3 kinase is also involved in regulation of the host innate immune response. The MK3 knockout mice differed in their expression and/or secretion of IFN gamma after influenza virus infection. Especially the number of IFN gamma expressing natural killer cells was increased in MK3 knockout mice compared to wt mice. *In vitro* studies using isolated T-cells strengthens the role of MK3 in the production of IFN gamma as cells deficient for MK3 produce a higher amount of IFN gamma after cytokine stimulation. This data suggests that MK3 is involved in regulation of IFN gamma.

Key words: Influenza, MAPKAPK3, IFNgamma, NK-cells, T-cells

P40

A reverse genetics approach to study the determinants for dsRNA-binding and PKR inhibition of the influenza A virus NS1 protein

K.L. Schierhorn[1*], T. Wolff[1]

[1]Robert Koch-Institute, Influenza and respiratory Viruses, Berlin, Germany

The non-structural protein 1 (NS1) of influenza viruses functions in inhibitingthe antiviral state in infected cells.While NS1 proteins of influenza A and Bviruses (A/NS1 and B/NS1, respectively)have only 20% sequence identity, they have a conserved RNA-binding domain and share functions as inhibition of the antiviral kinase PKR. We have previously shown that distinct basic amino acid residues of the B/NS1 protein are required for dsRNA-binding and silencing ofPKR activation.In contrast, the A/NS1 protein has been suggested to directly interact with PKR to inhibit its activation, however, leaving the contribution of dsRNA-binding of A/NS1 on PKR inhibition unclear.

Therefore, we performed a systematic analysis of basic amino acid residues inthe RNA-binding domain of A/NS1 using reverse genetics.Mutating basic amino acid residues in A/NS1 without affecting the nuclear localisation signal, we generated 8 mutant viruses having 1 or 2 amino acid exchanges.Three mutant A/NS1 proteins demonstrated a defect in dsRNA-binding. While 2 of the corresponding mutant viruses showed a 10-fold reduction in viral replication on alveolar epithelial cells, the third mutant virus showed 10000-fold reduction and a diminished inhibition of PKR activation.

Thus, this study emphasizes the impact ofdsRNA-binding of A/NS1 on viral replication. The effect ofdsRNA-binding of the A/NS1 protein on PKR inhibition and other dsRNA-dependent cellular factors is currently under investigation.

Key words: NS1, reverse genetics, PKR activation, RNA-binding (max. 5)

P41

The inhibition of human cytokines by small molecules such as RV1088 in response to influenza virus infections of primary respiratory cell cultures

J. Ashcroft[1*], D. Brookes[2] R. Cocking[1]K. Ito[2] and W. Barclay[1]

1 Section of Virology, Imperial College, London.
2. RespiVert Ltd., London.

Respiratory infections caused by influenza viruses are responsiblefor significant morbidity and mortality world-wide. Given the emergence of resistant strains to licensed antiviraldrugs including adamantanes and neuraminidase inhibitors, it is imperative that novel compounds be identified to treat influenza infections viruses.Severe outcome has been linked to the over induction of the host innate immune response resulting a “cytokine storm.” In certain patient populations, a therapeutic approach to help control over induction of the innate immune response may be of benefit.However concerns have been expressed that suppression of aspects of the host immune response might also lead to an increase in virus replication and directly enhance viral induced pathology. Using cultures of primary human airway epithelium, we have demonstrated that a small molecule developed by RespiVert (RV1088) can inhibit the induction of an array of human cytokines and at low multiplicities of infection, is also effective at reducing the replication of both type A and type B influenza strains. This or similar molecules, may represent a new generation of compounds suitable for the treatment of respiratory virus infections.

Key words: Influenza, Cytokines, Type A, Type B

P42

Innate resistance of pig cells against highly pathogenic avian influenza (HPAI) H5N1 virus infection is associated with reduced pro-inflammation and infectious virus release.

Rahul K. Nelli[1]*, Suresh V. Kuchipudi[1], Pengxiang Chang[1], Belinda P. Baquero[1], Amir Ghaemmaghami[2], Sharon Brookes[3],Ian H Brown[3], Stephen P. Dunham[1]and Kin-Chow Chang[1]

[1] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, College Road, Loughborough, Leicestershire LE12 5RD, UK.
[2] School of Molecular Medical Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.
[3] Virology Department, Animal Health Veterinary Laboratories Agency (AHVLA), Weybridge, Addlestone, Surrey KT15 3NB, UK

Dysregulated cytokine responseis regarded as a key contributor to the morbidity during 1918 H1N1 influenza pandemic and is also associated with poor prognosis during avian H5N1 influenza virus infections in humans. Interestingly, H5N1 infection of pigs causes no or only transient and mild pro-inflammatory cytokine response. To understand the molecular mechanismsinvolved in hypercytokinemiaduring H5N1 infections,we used primary human and pig respiratory epithelial cells and blood macrophages.Primary cells from both the species were equally susceptible to initial H5N1 virus infection.However, human cells showed high levels ofcytokine (TNF-α and IL6), and chemokine (CXCL9, CXCL10 and CXCL11) expression to H5N1 virus infection. Contrastingly, primary pig cells infected with H5N1 virus showed mild or no TNF-α and chemokine induction. The apparent lack of a strong pro-inflammatory response, corroborated by the absence of TNFα induction in H5N1 challenged pigs, coincided with reduced production of new infectious virus from infected pig epithelial cells. Cytokine stimulation rapidly induces, suppressor of cytokine signalling-3 (SOCS-3),a protein suppressor of the Jak/STAT pathway.In this study SOCS3 was highly expressed and transcriptionally up-regulated in H5N1 virus infected pig cells than human cells. Over-expression of SOCS3 in infected human macrophages dampened TNF-α induction. In summary, we found that reported low susceptibility of pigs to HPAI H5N1 coincides at the level of innate immunity of respiratory epithelial cells and macrophages with reduced viable virus output and an attenuated pro-inflammatory response, possibly mediated in part by SOCS3.

Key words: H5N1, TNF-alpha, pig, human, SOCS3 (max. 5)

P43

The importin-α7 gene is a determinant of viral cell tropism in the murine lung

P. Resa-Infante[1*], Gülsah Gabriel[2]

[1] Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

Influenza A viruses are a continuous threat to humans due to their ability to cross species barriers and adapt to new hosts. Since viral transcription and replication takes place in the nucleus of the host cell, the viral polymerase complex requires adaptive mutations in order to adapt to the mammalian nuclear import machinery upon avian-mammalian interspecies transmission. It has been shown that differential use of importin-α isoforms, a constituent of the classical nuclear import pathway, governs cell tropism and host adaptation of influenza viruses. While growth of highly pathogenic avian influenza viruses depends on importin-α3, growth of mammalian viruses depends on importin-α7 expression (Gabriel et al., 2011).
Here, we have performed a comparative analysis of the viral replication efficiency and cell tropism in the lungs of wildtype (WT) and importin-α7-knockout ($\alpha7^{-/-}$) mice using a recombinant H1N1 influenza virus which carries a GFP reporter gene in its NS segment (Manicassamy et al., 2010). We observed that upon GFP-virus infection, WT mice undergo a significant weight loss and 80% succumb to infection within 8 days, while $\alpha7^{-/-}$ mice lose less weight and present 100% survival. The analysis of viral kinetics in the lung revealed rapid extensive infiltration and destruction of the alveolar epithelium in WT mice. In contrast, lung tissues of infected $\alpha7^{-/-}$ animals were mostly intact with a preferential infection of the bronchial epithelium. Furthermore, higher levels of pro-inflammatory cytokines and chemokines were detected in WT than in $\alpha7^{-/-}$ mice. In summary, our findings strongly suggest that the importin-α7 gene plays a crucial role in the designation of viral cell tropism and pro-inflammatory immune response in the lung.

Key words: Pathogenesis, Host factors

P44

Inhibition of cellular MAP kinase p38 impairs influenza virus induced primary and secondary host gene responses and protects mice from lethal H5N1 infection

Börgeling, Y.[1*]; Schmolke, M.[1,2]; Viemann, D.[3,4]; Nordhoff, C.[1]; Roth, J.[3]; Ludwig S.[1]

[1]Institute of Molecular Virology (IMV), Centre for Molecular Biology of Inflammation (ZMBE), Westfälische Wilhelms-Universität Münster, Germany; [2]present address: Mount Sinai School of Medicine, Department of Microbiology, New York, USA; [3]Institute of Immunology, Westfälische Wilhelms-Universität Münster, Germany; [4]present address: Department of Pediatric Pulmonology, Allergology and Neonatology, MH Hannover, Germany

One characteristic of infections with highly pathogenic avian influenza viruses (HPAIV) is the cytokine burst that strongly contributes to viral pathogenicity. It has been suggested, that this hypercytokinemia is an intrinsic feature of infected cells and involves hyperinduction of p38 MAP kinase. Here we investigate the role of MAPK p38 signaling in the antiviral response in mice and endothelial cells, a primary source for cytokines during systemic infections.
Global gene expression profiling of HPAIV infected endothelial cells in presence of the p38 inhibitor SB202190 revealed, that inhibition of MAPK p38 leads to reduced expression of interferons (IFN) and other cytokines after H5N1 and H7N7 infection. More than 90 % of all virus induced genes were either partially or fully dependent on functional MAPK p38 signaling. This could be attributed to the fact that MAP kinase p38 inhibition not only affects primary gene expression responses to infection but also impairs the secondary gene expression response by interference with the JAK/STAT pathway.
In vivo inhibition of MAPK p38 leads to a nearly complete shutdown of virus induced cytokine expression concomitant with reduced viral titers, thereby protecting mice from lethal H5N1 infection.
These observations show, that MAPK p38 acts on two levels of the antiviral IFN response: Initially the kinase regulates IFN induction and later p38 controls IFN signaling and thereby expression of IFN-stimulated genes. Thus, inhibition of p38 may be an antiviral strategy that protects mice from lethal influenza via suppression of overshooting cytokine expression.

Key words: influenza A virus, MAPK p38, cytokine storm

P45

Decreased activity of avian-like influenza polymerase in human cells is independent of the viral nucleoprotein

Duncan Paterson*, Ervin Fodor

Sir William Dunn School of Pathology, University of Oxford, United Kingdom

Most avian influenza viruses are unable to replicate efficiently in human cells. This is partly due to the low activity of the viral RNA polymerase of avian influenza viruses in mammalian cells. An E to K adaptive mutation at residue 627 of the PB2 subunit of the polymerase is known to increase the activity of avian influenza virus polymerases in mammalian cells. Accordingly, viral ribonucleoprotein (RNP) reconstitution assays show that a viral polymerase containing PB2 627E characteristic of avian influenza viruses exhibits impaired activity in mammalian cells compared to a viral polymerase that contains PB2 627K characteristic of mammalian-adapted influenza viruses. In this regard, previous studies have shown that 627E inhibits the interaction of the polymerase with NP leading to speculation that avian virus derived RNA polymerases are impaired in RNP assembly and replication. This is in agreement with the observation that purified viral polymerases containing either PB2 627E or PB2 627K show a similar level of activity in transcription assays that require no RNP assembly. In order to address the mechanisms of impairment of PB2 627E in mammalian cells, we used a recently developed NP-independent cell based transcription/replication assay to assess viral polymerase activity. We found the PB2 627E polymerase was inhibited in mammalian cells suggesting that the reduced activity associated with PB2 627E is independent of NP. Moreover, these results demonstrate that reduced binding of NP by polymerases containing PB2 627E is unlikely to be responsible for the observed reduction in avian viral polymerase activity in human cells.

Key words: Influenza, RNA polymerase, PB2, host adaptation

P46

Avian and Human Influenza Virus Surveillance in Asian part of Russia (2010-2011)

Silko N.Yu.*, Sharshov K.A., Durymanov A.G., Sivay M. V., Saifutdinova S.G., Marchenko V.Yu., ,2Kurskaya O.G., Sobolev I.A., Susloparov I.M., Zaikovskaya A.V., Alekseev A.Yu., Ilyicheva T.N., Shestopalov A.M.

State Research Center of Virology and Biotechnology «VECTOR»

During surveillance study in 2010-2011 we collected 2620 samples from wild water birds in the three regions (Novosibirsk Region, Omsk Region and Altai Region) of the Western Siberia. The samples were tested by standard methods and 41 AIVs were isolated (isolation rate is 1.56%). AIV strains have the following subtypes: H3N8 (n=12), H3N2 (n=2) ; H3N3 (n=1), H4N6 (n=3); H1N1 (n=1); H8N4 (n=1), H5N3 (n=1). 20 viruses were not identified and are studied now. During surveillance study in 2010-2011 more than 5000 samples from different bird species were collected in Russian Far East. We isolated 43 viruses from 9 bird species. AIV strains had the following subtypes: H1N? (n=1), H3N6 (n=5), H3N2 (n=1), H3N? (n=4), H6N? (n=4), H4N? (n=2), H6N6 (n=3), H3N? (n=4), H?N1 (n=1), H4N1 (n=1), H3N8 (n=3), H13N6 (n=4). 10 viruses were not identified and are studied now. Isolation rate is near 0,65%. Phylogenetic analysis of the M gene of isolated viruses revealed different lineages of influenza viruses isolated and some evolutionary trends are discussed. We isolated 48 Human influenza viruses during 2010-2011 in Asian part of Russia: thirty eight A/H1N1 2009, three A/H3N2 and seven influenza B isolates. The A/H1N1 2009 viruses had sporadic nucleotide substitutions in genome and antigenically were equal to the vaccine strain A/California/07/09 (H1N1 2009). But isolates 2011 had more low HA titer with chicken, goose and guinea pig red blood cells compared to the strains, isolated in this region during 2009-2010. The A/H3N2 viruses were antigenically related to A/Perth/16/2009, influenza B isolates were shown to be B/Vic-lineage viruses.

P47

Evaluation of antiviral activity of SALPs against influenza viruses

Julia Hoffmann*, Klaus Brandenburg[2] and Gülsah Gabriel[1]

[1]Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany, [2]Leibniz-Center for Medicine and Biosciences, Research Center Borstel, Borstel, Germany

Influenza A viruses are a continuous threat to humans as recently illustrated by the 2009 H1N1 pandemic. With increasing resistance of circulating influenza virus strains to currently available drugs, the development of novel antivirals is urgently needed. Here, we have evaluated a series of synthetic anti-lipopolysaccharide peptides (SALPs), previously shown to inhibit viral attachment and entry of several enveloped viruses into the host cell by binding to heparan sulfate as cellular docking molecule, for their antiviral activity against influenza A viruses. We found that SALPs are able to inhibit human H3N2 and mouse-adapted H7N7 influenza virus replication by 1-4 logs in a dose-dependent manner. Furthermore, our analysis revealed that some of these SALPs specifically bind sialic acids with high affinities. Thus, in addition to heparan sulfate, SALPs are also able to bind sialic acids and thereby inhibit influenza virus infection *in vitro*. Next, we assessed the inhibitory effect of the SALPs in mice. All animals, which have been simultaneously inoculated with a mouse-adapted H7N7 virus and the given SALPs, survived with significantly reduced virus lung titres an otherwise 100% lethal infection in untreated mice. Taken together, we could show that SALPs are promising novel candidates to target influenza virus replication *in vitro* and *in vivo*.

Key words: synthetic anti-lipopolysaccharide peptides (SALPs), Influenza A virus infection, antiviral activity

P48

The influenza virus and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in the human respiratory and gastrointestinal tracts

S. Bertram[1*], A. Heurich[1], H. Lavender[2], S. Gierer[1], S. Danisch[3], P. Perin[3], J.M. Lucas[4], P.S. Nelson[4], E.J. Soilleux[5], S. Pöhlmann[1]

[1]German Primate Center, Göttingen, Germany; [2]Oxfabs, Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University Oxford, U.K.; [3]Institute of Virology, Hannover Medical School, Hannover, Germany; [4]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; [5]Department of Cellular Pathology and Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, University of Oxford, Oxford, U.K.

Influenza viruses and SARS-coronavirus (SARS-CoV) require proteolytic activation by host cell proteases for infectious entry into target cells. Two members of the type II transmembrane serine protease (TTSP) family, TMPRSS2 and HAT, were shown to activate influenza A viruses (FLUAV) and SARS-CoV in cell culture. However, the expression of these proteases in potential target cells in human tissues has not been systematically analyzed. Employing immunohistochemistry, we found that TMPRSS2 and HAT are coexpressed with 2,6-linked sialic acid, the major receptor determinant of human FLUAV, throughout the respiratory tract. Similarly, ACE2, the SARS-CoV receptor, and TMPRSS2 were coexpressed on several sites of the upper and lower aerodigestive tract, including type II pneumocytes, the major viral target cells. Furthermore, we could show that TMPRSS2 of porcine and avian origin can activate FLUAV, indicating that this protease could promote viral spread in species integral to the influenza virus zoonosis. In addition, TMPRSS2 from mice, which represent an important animal model for influenza virus infection, was able to activate FLUAV, indicating that mice are an adequate model system to study the importance of TMPRSS2 for viral spread and pathogenesis. In summary, our results demonstrate that TMPRSS2 and HAT are expressed on important FLUAV and SARS-CoV target cells and could therefore support viral spread in the human host.

Key words: influenza A virus, entry, type II transmembrane serine proteases, TMPRSS2, HAT

P49

Influenza A virus nucleoprotein targets specific subnuclear domains

Chris T. Hoefer [1*], Michael Veit [2], Andreas Herrmann [1]

[1] Humboldt-Universität zu Berlin, Department of Biology, Molecular Biophysics, Berlin, Germany; [2] FU Berlin, Faculty of Veterinary Medicine, Institute of Immunology, Berlin, Germany

The Influenza virus nucleoprotein (NP) is the major structural protein of the genomic viral ribonucleoprotein complexes. While it is known that NP localizes in the nucleus when expressed alone, its heterogeneous subnuclear distribution is less well characterized.
During the last years, our conception of nuclear organization and its spatial and functional compartmentalization has expanded enormously. For various viruses, it has been reported that viral proteins target subnuclear structures as a mechanism to interfere with host cell antiviral activity [1] or to localize their genomes to specific domains for efficient viral replication [2]. In case of Influenza virus, it was previously demonstrated that proteins M1, NS1 and NEP associate with PML nuclear bodies and that number and size of different types of nuclear bodies are altered during infection [3].
We show that NP is locally enriched in the nucleus in the absence of other viral proteins and displays low mobility in different subnuclear structures indicating an intrinsic affinity for these nuclear domains. Identification of subnuclear structures accessed by NP will provide information on functional processes and cellular machinery targeted by the protein. We studied subnuclear accumulations of NP microscopically by fluorescence photoactivation and immunofluorescence for colocalization analysis using different NP mutant constructs. Our results show that NP associates with high affinity with nucleoli and subnuclear structures that are frequently found adjacent to PML and Cajal Bodies. We hypothesize that the NP specifically targets these domains, either linking specific cellular machinery to viral replication or interfering with host cell processes.

Key words: Influenza A nucleoprotein, nuclear bodies

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P50

Cloning, Expression, and Characterization of Ferret Genes

G. Manukian[1], S. Naveen[1], BS Kaplan[2], TG Voss[1*]

[1]Tulane University School of Medicine, Department of Microbiology and Immunology, New Orleans LA USA
[2]Department of Infectious Diseases, St. Jude Children's Research Hospital
Memphis, TN USA

Due to a paucity of reagents for study immune and other relevant cellular processes in the ferret, we have focused considerable effort in the development of ferret specific reagents for cell surface molecules, cytokines, and genes of interest in the adaptive and innate immune response to infection. Here we present results for the cloning and expression of FoxP3, CD25, IL10, IL17f, and CRM-1 from primary ferret tissues. Expressed proteins were confirmed by Western blot, Immune fluorescence, and functional assays.Our results indicate successful cloning and expression of FoxP3, CD25, IL10, IL17f, and CRM-1 from the ferret. Functional studies with CRM-1 include use of ferret CRM-1 in a two hybrid system (mammalian) to show interaction with influenza A Nuclear Export Protein (NEP), the viral protein involved in nuclear export of vRNP. For FoxP3, his-tagged protein was identified by Western blot and purified after cleavage of aenterokinasethioredoxin tag. An HIV-tat peptide containing FoxP3 was used for functional studies in cells that were transduced with native FoxP3. Ferret CD25 was purified and identified by Western blot. Proinflammatory or regulatory cytokines (IL-10 and IL-17) function was assayed using primary ferret PBMC and expression of these cytokines in ferrets was quantified by qRT-PCR during the course of infection. These tools will allow expansion of the ferret model for influenza and other relevant human infectious disease. We are currently examining the role of T-reg in pregnancy-associated mortality in influenza infection in this model.

P51

Antiviral defense mechanisms in alveolar type II epithelial cells are altered in chronically diseased lungs and counteract with influenza A infection

Marcus Gereke[1,2*], Lisa Andzinski[1], Dorthe von Smolinski[3], Achim Gruber[3], Andrea Autengruber[2], Harro Frauendorf[1] and Dunja Bruder[1,2]

[1]Helmholtz Centre for Infection Research, Braunschweig
[2]Institute of Medical Microbiology, Otto-von-Guericke University, Magdeburg
[3]Department of Veterinary Pathology, Free University Berlin, Berlin

It has been widely discussed that acute respiratory infections are linked to the exacerbation of chronic inflammatory diseases or autoimmune disorders.
To study the influence of influenza A virus (IAV) infection on mice suffering from a chronic lung inflammation caused by alveolar type II epithelial cell (AECII) self-antigen recognition by autoreactive CD4+ T cells, we infected chronically diseased SPC/HAxTCR/HA and healthy TCR/HA control mice with IAV A/PR/8/34/H1N1. Surprisingly, IAV infection in SPC/HAxTCR/HA suffering from autoimmune lung disease did not result in IAV specific weight loss or histological signs of IAV specific pneumonia compared to controls. Furthermore we observed a drastically reduced viral load in lungs of SPC/HAx/TCR/HA mice compared to TCR/HA mice after infection. These findings suggest early acting antiviral mechanisms interfering with a reproductive viral infection in the lung. Since AECII are one of the major target for influenza A infection we characterized in detail AECII derived from mice suffering from chronically inflamed lungs. In comparison to AECII from healthy control mice we observed elevated antiviral gene expression (of e.g. the ubiquitin-like protein ISG15) and less viral replication in *in vitro* infections with IAV.
In contrast to our initial assumption that secondary infection by IAV leads to a breakdown of tolerance and exacerbation of lung inflammation SPC/HA/TCR/HA mice suffering from chronic pulmonary disease these mice establish antiviral and well balanced immunological mechanisms to counteract viral infections. Therefore, our data emphasize that the lung epithelium plays an important role in antiviral responses to orchestrate the antiviral immunity in the host.

Key words: influenza infection, antiviral response, alveolare type II epithelial cell (AECII), chronic lung disease

P52

Early protection of balb/c mice against lethal HPAIV H5N1 challenge infection

Susanne Röhrs*, Donata Kalthoff, Martin Beer

Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17498 Greifswald-Insel-Riems

The primary hosts of highly pathogenic avian influenza (HPAIV) H5N1 viruses are birds, however, this viruses have also the potential to infect mammalians including humans. Therefore, virus-host interactions should be also studied in mammalian hosts, especially concerning the immune response following vaccination or challenge infection.
In our study we used a novel neuraminidase-deleted apathogenic variant of the HPAIV H5N1 strain R65 ("H5N1del") as a vaccine model for a series of immunization/challenge experiments. In a first trial, 6 week old balb/c mice were immunized with a single dose of 104.5 TCID50, and it could be demonstrated that the animals did not show any clinical signs post vaccination and no viral RNA could be detected in organ samples, demonstrating that the neuraminidase-deleted strain was innocuous for mice. In a second trial we intraperitoneally immunized 6 week old balb/c mice 1, 3, 7, 14 and 21 days before a lethal HPAIV H5N1 challenge infection with a single dose of the H5Ndel mutant. Interestingly, the immunization could not completely prevent clinical symptoms, but all animals survived the infection, although only the mice immunized 14 and 21 days before infection had high titers of hemagglutinin-specific antibodies.
In a third trial 6 week old balb/c mice were immunized twice with the inactivated H5Ndel together with an adjuvant. Surprisingly all animals died as fast as the infection control animals although these group had high titers of hemagglutinin-specific antibodies.
In conclusion, a very early onset of immunity against HPAIV seems feasible also in a mammalian infection model using a modified live virus variant. This data should be taken into consideration for future vaccine developments and control strategies.

Key words: HPAIV; balb/c mice, immunization, immune response

P53

Influenza virus tropism in human and swine respiratory tract

Renee WY Chan[1,2*], Michael CW Chan [1], Icarus WW Chan[1], John Nicholls[2], and Malik Peiris[1]
[1]Centre of Influenza Virus Research and School of Public Health, [2]Department of Pathology, Li Ka Shing Faculty of Medicine, University of Hong Kong.

Influenza A virus, is both a major pandemic concern, as well as inflicting a huge loss in the poultry industry. Human and pig are the main hosts of influenza A viruses and the virus can infect and established its lineage within these hosts. Cells culture and animal models have been used to study the pathogenesis of the influenza disease although many of the factors leading to disease progression still remain unsolved. Human subject is the most accurate reflector of human disease, however, many experiments cannot be carried out ethically in humans. Hence, in vivo animal model is commonly adopted. However, there are huge differences in the anatomy, physiology and immune response between animal models and that of humans and this still leads to a major discrepancy to humans. As a result, the studies using ex vivo organ culture models, which retain a high relevance to in vivo human physiology, become important and valuable.

Our group has developed human and swine respiratory organ explant cultures and characterized their influenza virus receptor profile. We used these upper, conducting and lower respiratory epithelial cultures to investigate the virus tropism of seasonal influenza, pandemic influenza and highly pathogenic influenza virus in earlier years. With high correlation to ferret transmission model, we hypothesized that virus which can transmit efficiently between humans replicated well in the human upper airways.

The human health risk of newly isolated influenza virus strains from surveillance programs will be assessed and discussed by evaluating the virus tropism in these explant cultures of human respiratory tissue.

Key words: Influenza virus, human respiratory culture, swine respiratory culture, tissue tropism

P54

A systems-based approach to examine the host response in permissive cells types infected with Low Pathogenic Avian Influenza Viruses and low passage human clinical influenza pH1N1 isolates.

Richard Sutejo[1], Dawn S. Yeo[1,2], Myint Zu Myaing[1],Chen Hui[1] ,Jiajia Xia[1], Debbie Ko[1], Peter C. F. Cheung[1], Boon-Huan Tan[2] and Richard J. Sugrue[1*]

(1) Division of Molecular Genetics and Cell Biology, School of Biological Sciences, Nanyang Technological University, Singapore, Singapore (2) Detection and Diagnostics Laboratory, DSO National Laboratories, Singapore, Singapore

In this study we used a using a systems-based approach to examine the host response in permissive cell types infected with human and avian viruses that have been isolated in Singapore. The host response to the low pathogenic avian influenza (LPAI) H5N2, H5N3 and H9N2 viruses were examined in A549, MDCK, and CEF cells. The H5N2 and H5N3 viruses replicated efficiently in A549 and MDCK cells, while the H9N2 virus replicated least efficiently in these cell types. However, all LPAI viruses exhibited similar and higher replication efficiencies in CEF cells. A comparison of the host responses of these viruses and the H1N1/WSN virus and low passage pH1N1 clinical isolates was performed in A549 cells. The H9N2 and H5N2 virus subtypes exhibited a robust induction of Type I and Type III interferon (IFN) expression, sustained STAT1 activation from between 3 and 6 hpi, which correlated with large increases in IFN-stimulated gene (ISG) expression by 10 hpi. In contrast, cells infected with the pH1N1 or H1N1/WSN virus showed only small increases in Type III IFN signalling, low levels of ISG expression, and down-regulated expression of the IFN type I receptor. JNK activation and increased expression of the pro-apoptotic XAF1 protein was observed in A549 cells infected with all viruses except the H1N1/WSN virus, while MAPK p38 activation was only observed in cells infected with the pH1N1 and the H5 virus subtypes. No IFN expression and low ISG expression levels were generally observed in CEF cells infected with either AIV, while increased IFN and ISG expression was observed in response to the H1N1/WSN infection. These data suggest differences in the replication characteristics and antivirus signalling responses both among the different LPAI viruses studies, and between these viruses and the H1N1 viruses examined. These virus-specific differences in host cell signalling highlight the importance of examining the host response to avian influenza viruses that have not been extensively adapted to mammalian tissue culture.

P55

Activation of the Rar-related orphan receptor alpha by H5N1 leads to subsequent NF-κB inhibition and suppression of inflammatory responses in monocytes

J. Friesenhagen[1,2*], D. Viemann[1], Y.Boergeling[3], M. Schmolke[3], C.O. Spiekermann[2], S. Kirschnek[4], S. Ludwig[3] , J. Roth[2], D. Viemann[1]

[1]Medical School Hannover, Experimental Neonatology, Hannover, Germany; [2]University of Muenster, Institute of Immunology, Muenster, Germany; [3]University of Muenster, Institute of Molecular Virology, Muenster, Germany; [4]University of Freiburg, Freiburg, Germany

Infections of the human host by highly pathogenic avian influenza viruses (HPAIV) are characterized by development of a cytokine storm and systemic spreading of infection. We assumed monocytes as main producers of cytokines to be involved in the emergence of a cytokine burst. Surprisingly, a genome-wide microarray analysis showed a strongly reduced inflammatory response of human blood-derived monocytes infected with highly pathogenic avian influenza virus (HPAIV) A/Thailand/1 (KAN-1)/2004 (H5N1) compared to monocytes infected with low pathogenic human influenza virus A/PR8/34 (H1N1). Transcription factor profiling revealed overexpression of the Rar-related orphan receptor alpha (RORα), a nuclear receptor known to suppress NF-κB signalling. Western Blot analysis confirmed a stronger activation and nuclear translocation of RORα in case of H5N1 infection compared to H1N1 infection in human blood-derived monocytes. In line with this, translocation of the NF-κB subunit p65 was clearly reduced during H5N1 infection. The establishment of an ER-Hoxb8 RORα knockout cell line derived from embryonic liver stem cells of RORα$^{-/-}$ fetuses enabled us to prove coherence between RORα activation and NF-κB inhibition. Additionally, we could show a biological relevance of RORα activation during H5N1 infection as we observed a significantly stronger inflammatory and antiviral response in RORα knockout monocytes compared to corresponding Wt cells. In summary, we present a novel mechanism by which H5N1 escapes the first immune reponse. This mechanism contributes to the high pathogenicity of H5N1 by enabling the virus to spread systemically.

Key words: transcription factors, innate immunity, highly pathogenic avian influenza viruses

P56

A siRNA screen to detect influenza virus strain-specific differences in required host cell factors

M. Lesch[1*], P. Braun[1], T.F. Meyer[1], A. Karlas[1]

[1]Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany

Influenza viruses (IV) depend on the host cellular machinery to complete their life cycle. Thus, host factors might be attractive targets for antiviral compounds. To identify cellular genes required for IV infection, six genome-wide screens have been performed by independent laboratories, and 1449 human genes have in total been proposed as host dependency factors for IV replication. The strains applied in these studies had in common that they originated from seasonal human influenza viruses of genus A. Thus, it is unclear if the published factors are also required for influenza virus strains with a genotype and phenotype differing from that of the previously employed isolates. This information is, however, essential for the development of a broadly active drug. Therefore, we will perform a short interfering RNA (siRNA) screen using six IV strains differing in their genetic and phenotypic properties (Spanish influenza, H5N1 avian influenza, swine-origin influenza, influenza B, contemporary seasonal influenza A, A/WSN/33). The genes to be screened comprise 352 published host dependency factors as well as the human kinome (719 genes). This study will help to unravel IV strain-specific differences in the reliance on host cell genes and may reveal novel options for antiviral therapy.

Key words: screen, strain-specific, host factor, Spanish flu, H5N1

P57

Use of CaCo-2 cells for isolation of influenza virus from clinical and post mortem samples

E. Bildanova*, D. Danilenko, T. Smirnova, T. Gudkova, M. Yu. Eropkin

[1]Research Institute of Influenza, Ministry of Health and Social Development, Russian Federation

CaCo-2 cell line was originally obtained from human colon carcinoma and for the first time was used for influenza virus isolation in 1998. Since then it has been shown that these cells are capable to perform intracellular proteolysis of influenza hemagglutinin and thus do not require external trypsin addition for influenza virus replication.
CaCo-2 cell culture was tested along with MDCK line for isolation of influenza H1N1pdm and B strains from clinical specimens and post-mortem materials. The infectious activity of influenza viruses in CaCo-2 cell line was practically the same as in MDCK cell line. The rate of viral isolation was practically identical for both cell lines tested. 30 PCR positive for H1N1pdm clinical samples and 30 post-mortem materials were used to perform parallel isolation in CaCo-2 and MDCK. 10 virus isolates were obtained from MDCK and 9 from CaCo-2 from swabs. Only 4 H1N1 pdm viruses could be isolated from post-mortem materials, all of them from CaCo-2 cells. Viral isolation rate for influenza B viruses was also evaluated and turned out to be practically the same for both of cell lines: for the 30 PCR positive clinical samples influenza B viruses were isolated in 28 cases for MDCK cell line and 26 for CaCo-2.
Overall, CaCo-2 line can be a valuable cell line for virological research, particularly in the cases when the virus in poorly isolated in MDCK cells, such as H1N1 pdm influenza from post-mortem materials.

Key words: CaCo-2, influenza isolation, pandemic influenza

P58

Effect of influenza virus infection on gelatinases in human monolayer cell cultures

D. Danilenko*, M. Dyukov, T. Smirnova

Research Institute of Influenza, Ministry of Health and Social Development, Russian Federation

Matrix metalloproteinases (MMPs) represent a large family of zinc-dependent endopeptidases that are capable to degrade extracellular matrix proteins. They are thought to play an important role in processes of cell proliferation, migration, differentiation, apoptosis. All MMPs are synthesized as zymogens and require extracellular activation by proteases or other agents. We investigated the impact of influenza infection on gelatinases by means of gel zymography. Two human monolayer cell cultures were used: A-549 (lung carcinoma) and ECV-304 (endothelial line). We compared the effect of human and avian viruses on MMP induction. A-549 showed rather poor MMP-2 and MMP-9 activity in control samples and in virus infected samples. No clear difference could be revealed by zymography analysis. ECV-304 cell line showed intense bands for MMP-9 and rather weak for MMP-2 in control samples. When infected with different influenza strains the intensity of the bands didn't vary substantially from the bands in the control samples but we observed an interesting phenomenon. MMP-9 bands in the samples that were infected with influenza virus demonstrated reduction in their molecular weight. This reduction was the most pronounced in the case of H5N1 strain, a bit less intensive for H1N1pdm strain and only slight for seasonal H3N2 strain. Thus, the presence of the virus led to alteration of MMP-9 molecular weight possibly by additional cleavage of the pro-enzyme. None of the antiviral drugs had any effect on either the apparent band intensity or molecular weight change pattern described before. The MMP2 band appeared unchanged being of fairly low intensity in all ECV-304 samples.

Key words: influenza, matrix metalloproteinases, gelatinases, MMP-9

P59

Acylation of influenza A virus M2 and HA might work synergistically for efficient budding of virus particles

S. Siche[1*], B. Thaa[1], L. Möller[2], Andreas Herrmann[3], M. Veit[1]

(1) Freie Universität Berlin, Fachbereich Veterinärmedizin, Institut für Immunologie und Molekularbiologie, Philippstraße 13, 10115 Berlin, Germany
(2) Robert-Koch-Institut, Nordufer 20, 13353 Berlin, Germany
(3) Humboldt-Universität zu Berlin, Institut für Biologie, Molekulare Biophysik, Invalidenstraße 42, 10115 Berlin, Germany

Assembly and budding of influenza virus is a complex multi-step process occurring at cholesterol-rich domains of the plasma membrane ("membrane-rafts").The proton channel protein M2 is implicated in virus pinching-off, probably by wedge-like membrane insertion of its amphiphilic helix located in the cytoplasmic tail. Palmitoylation and cholesterol binding motifs in this helix are assumed to target M2 to the edge of rafts. However, recombinant viruses carrying mutations in these features do not show any effect on replication in cell culture.
During budding M2 clusters with hemagglutinin (HA), dependent on acylation of HA at 3 conserved cysteines: Ac1 (transmembrane domain), Ac2 and Ac3 (cytoplasmic region). The fatty acids contribute to raft targeting of HA.
To investigate a functional coupling of HA's and M2's acylation for virus budding we performed reverse genetics in the WSN background. Recombinant viruses with disrupted acylation sites in the cytoplasmic tail of HA (Ac2 and Ac3) could not be rescued. The Ac1 mutant showed a severe growth defect in MDCK-cells, the titre was reduced by 4 logs. A further reduction by 4 logs occurred in the Ac1+M2 mutant, although deletion of the acylation site in M2 alone had no effect. Electron microscopy revealed a tendency of Ac1+M2 to produce more filamentous virus forms and "daisy chain" particles.
Hence, mutation of M2 aggravates the effect of disrupting the Ac1 acylation site in HA indicating that M2- and HA-bound fatty acids work synergistically. Ongoing studies to determine the protein composition and fusion activity of recombinant viruses will clarify the functional significance.

Key words: HA, M2, acylation

P60

Distinct gene loci control the host response to influenza H1N1 virus infection in a time-dependent manner

H. Kollmus[1*], T. Nedelko[1], F. Klawonn[2], S. Spijker[3], Lu Lu[4,5], R. Alberts[1], R. W. Williams[4], K. Schughart[1]

[1]Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, and University of Veterinary Medicine Hannover, Germany; [2]Department of Bioinformatics and Statistics, Helmholtz Centre for Infection Research, Braunschweig, Germany; [3]Department of Molecular and Cellular Neurobiology, Neuroscience Campus Amsterdam, VU University, Amsterdam, The Netherlands; [4]Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, USA; [5]Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong, China

There is strong evidence that genetic factors modulate the severity of influenza infection in humans. Using the mouse as model organism for influenza A infection, we previously demonstrated that the genetic background has a very strong influence on the host response to influenza A infection. In particular, strain C57BL/6J, the most widely used strain of mouse in biomedical research, is comparatively resistant. In contrast, DBA/2J is highly susceptible.
To map regions of the genome responsible for differences in influenza-susceptibility we infected 55 BXD-type lines derived from a cross between C57BL/6J and DBA/2J strains with influenza A virus (PR8, H1N1). We monitored body weight, survival, and mean time to death for 13 days post infection and used these traits for QTL (quantitative trait loci) mapping with tools provided by GeneNetwork. QTL mapping revealed QTLs on chromosomes 2, 5, 16, 17, and 19 influencing the host response to influenza infection. Body weight and survival QTLs showed a time-dependent profile indicating that different genetic factors are important for the host response in a temporal dynamic manner after infection. Analysis of multiple gene expression data sets in these same strains highlighted about 30 strong candidate genes in the respective QTL intervals that may control influenza A susceptibility and resistance. We describe these candidate genes and their presumed biological functions during the infection process.

Key words: Influenza A, host susceptibility, QTL mapping, mouse inbred strains, virus host interaction

P61

Novel influenza antiviral agents

O. Denisova*, L. Kakkola, D. Kainov

Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Finland

To ensure efficient production of infectious viral particles influenza A virus subverts different cellular functions. These functions could be perturbed by small molecules to inhibit influenza A virus infection. Here, we report 2 novel and 1 known host-directed compounds that inhibit influenza A virus infection *in vitro* and *ex vivo* at noncytotoxic concentrations. These compounds also inhibit other virus infections suggesting that these anticancer agents target cellular functions that are efficiently exploited by different viruses. Our results could provide a foundation for the development of novel antiviral therapeutics.

Key words: small molecules, virus entry

P62

Porcine airway epithelial cells provide a model system to study proteolytic activation of influenza A viruses

C. Freuer*, H.-D. Klenk, W. Garten, E. Böttcher-Friebertshäuser

Institute of Virology, Philipps University Marburg, Hans-Meerwein-Str. 2, 35043 Marburg

Influenza A viruses cause an acute respiratory disease and are responsible for recurring seasonal epidemics and occasional pandemics in humans. Pigs are natural hosts of influenza A viruses as well. Due to their susceptibility to both avian and human influenza viruses, swine may serve as intermediate hosts and thus play an important role in the epidemiology of influenza A viruses. As in humans, respiratory epithelial cells are the primary host target cells for influenza A viruses in swine. Hence, it can be assumed that the very same HA-activating mechanism is accomplished by porcine proteases homologous or similar to human HA-processing proteases. Here, we analysed proteolytic activation of influenza A virus HA in primary porcine airway epithelial cells. Seasonal and pandemic influenza A viruses replicated efficiently in primary porcine respiratory epithelial cells in the absence of exogenous HA-activating proteases. Interestingly, the porcine protease pTMPRSS2, a protease homologous to human HA-activating protease TMPRSS2, was found to be expressed in several sections of the porcine respiratory tract. We cloned pTMPRSS2 from cultures of primary bronchial epithelial cells (PBEC) and demonstrated HA cleavage and proteolytic activation of influenza A viruses by pTMPRSS2.
In conclusion, pTMPRSS2 is a promising candidate for proteolytic activation of influenza A virus HA in the respiratory tract of pigs and, moreover, primary porcine airway epithelial cells constitute a valuable in vitro model system for influenza A virus infections.

Key words: hemagglutinin, proteolytic activation, primary porcine airway epithelial cells

P63

A four-year survey of the oseltamivir-resistant influenza viruses in Ukraine

L. Leibenko[1*], S. Babii[1], N.Kurinko[1], A. Mironenko[1]

[1]L.V. Gromashevsky Institute of Epidemiology & Infectious Diseases AMS of Ukraine, Kiev, Ukraine

The neuraminidase (NA) inhibitor oseltamivir is currently an important antiviral drug for the treatment of seasonal and pandemic influenza. Antiviral resistant of influenza viruses has become an important issue for the study since 2007-2008 when were distinguished the many oseltamivir-resistant A(H1N1) viruses all over the world. All NA inhibitor-resistant influenza viruses characterized to date have contained specific mutations in the NA molecule, that are NA subtype specific and differ in accordance with the NA inhibitor used.
The aim of our work was determine the rate of oseltamivir resistance among influenza A and B isolates in Ukraine during the 2007-2011 influenza seasons.
In this reason, we conducted a retrospective analysis of laboratory confirmed influenza cases in Ukraine. Oseltamivir resistance, determined both by neuraminidase inhibition assay and by detecting the H275Y mutation in the neuraminidase (NA) gene of A(H1N1) viruses, was observed in 36% (9 of 25) of isolates from 2007-2008 influenza season. Among isolates tested for their susceptibility to neuraminidase inhibitors in 2008-2009 (7 viruses of influenza B, 29 — pandemic influenza A(H1N1) viruses); in 2009-2010 (9 — influenza A(H3N2) viruses) and in 2010-2011 (2 — influenza A(H3N2) viruses, 18 — influenza B and 3 — pandemic influenza A(H1N1) viruses), all 100% (68 of 68) were sensitive to oseltamivir.
The epidemiological data shoved, that drug resistance to oseltamivir has been detected mainly in young children of age groups 0-4 (20%), 5-9 (30%) and 10-14 (50%) years.
Timely monitoring and reporting of antiviral drug-resistance is important at the global, national, and the local community levels.

Key words: influenza, neuraminidase inhibitor, oseltamivir resistance

P64

Whole-genome transcriptome analysis of influenza-infected mouse lungs reveals the kinetics of the complex host response

Esther Wilk[1*], Claudia Pommerenke[1], Barkha Srivastava[1], Konstantina Dimitrakopoulou[2], Anastasios Bezerianos[2], Robert Geffers[3] and Klaus Schughart[1]

[1]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany and University of Veterinary Medicine Hannover, [2]School of Medicine, University of Patras, Patras, Greece; [3]Research Group Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig Germany;

To capture the kinetics of the host response after influenza infection, we studied the transcriptome in mouse lungs after infection with an H1N1 influenza A virus (PR8) using Agilent´s microarrays. Global analyses as well as cell specific gene profiles were applied. Flow cytometry and immunohistology were performed to correlate gene expression to the cellular immune response. Distinct phases of the host response were observed in C57BL/6J mice after infection with a non-lethal dose of PR8. The increase in interferon genes and upregulation of a defined NK-specific gene set revealed the initiation of the innate immune response phase. Infiltration and activation of T and B cells could be seen by an augmentation of T and B cell specific expression signatures. Our results demonstrated that the permanent changes in expression profiles were still evident at 60 days after infection. Furthermore, we performed a network analysis based on the kinetics of gene expression changes and established a model which also includes genes that are not well studied or have not yet been annotated. We also compared the expression profiles from wild type with $Rag2^{-/-}$ mice, demonstrating that the deficiency of T and B cells in $Rag2^{-/-}$ mice is well reflected within the transcriptome. Finally, we compared the gene expression from resistant C57BL/6J mice to the susceptible DBA/2J mouse strain. In conclusion, our comprehensive gene expression study describes the entire host response to an acute influenza A infection at the transcriptome level suitable to unravel the biological mechanisms for phenotypic alterations in genetically different mice.

Key words: Whole-genome expression studies, mouse model, network analysis

P65

A network of intermolecular interactions between viral RNA segments of an avian H5N2 influenza A virus: comparison with a human H3N2 virus

Catherine Isel[1*], Cyrille Gavazzi[1], Emilie Fournier[1], Vincent Moulès[2], Daniel Thomas[3], Bruno Lina[2] and Roland Marquet[1]

[1]Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084 Strasbourg, France; [2]Virologie et Pathologie Humaine, Université Lyon 1, FRE 3011 CNRS, Faculté de Médecine RTH Laennec, 69008 Lyon, France; [3]Interactions Cellulaires et Moléculaires, Université Rennes 1, UMR 6026 CNRS, Campus de Beaulieu, bâtiment 13, 35042 Rennes, France.

The genome of influenza A viruses (IAV) is split into eight negative strand viral RNAs (vRNAs) that are packaged as viral ribonucleoproteins (vRNPs). The existence of a segment-specific packaging mechanism is well established but its molecular basis remains to be deciphered. Selective packaging could be mediated by direct interactions between the vRNA packaging regions, but such interactions have never been demonstrated in virions. Recently, we showed that the eight vRNAs of a human A/Moscow/10/99 (H3N2) IAV (MO) form a single interaction network *in vitro* that involves regions of the vRNAs known to contain packaging signals in the most studied human H1N1 IAV strains. Here, we show that the eight vRNAs of an avian A/Finch/England/2051/92 (H5N2) IAV (EN) also form a single network of interactions *in vitro* but interestingly, the interactions and the regions of the vRNAs they involve differ between the MO and EN viruses. The vRNA sequences involved in five of these interactions were identified, at the nucleotide level, and in one case, we validated the existence of the interaction using silent compensatory mutations in the interacting sequences. Electron tomography also revealed significant differences in the interactions taking place between vRNPs in EN and MO virions, despite a canonical "7+1" arrangement of the vRNPs inside the viral particles.

Key words: Influenza A, RNA packaging, vRNA, vRNP

P66

Small molecule inhibitors of the c-Jun N-terminal kinase (JNK) possess antiviral activity against highly pathogenic avian and human pandemic influenza A viruses

Wolfgang Nacken*, Christina Ehrhardt and Stephan Ludwig

Institute of Molecular Virology (IMV), Centre of Molecular Biology of Inflammation (ZMBE), Westfaelische-Wilhelms-University Muenster, Muenster, Germany

C-Jun N-terminal kinases (JNK) are activated in course of many viral infections. Here, we analysed the activity of JNK inhibitors on influenza A virus (IAV) amplification. Human lung epithelial cells were infected with either the highly pathogenic avian virus strain A/FPV/Bratislava/79 (H7N7) or the pandemic swine-origin influenza virus A/Hamburg/4/09 (H1N1v). The application of the JNK inhibitors SP600125 and AS601245 reduced IAV amplification by suppressing viral protein and RNA synthesis. While AS601245 appeared to generally block the transcription of newly introduced genes, SP600125 specifically affected viral RNA synthesis. Overexpression of a dominant negative mutant of the JNK activating kinase SEK/MKK4 (mitogen-activated protein kinase kinase 4) and siRNA mediated suppression of JNK2 expression confirmed that specific manipulation of the JNK pathway attenuates virus propagation. An IAV minigenome replication assay revealed that SP600125 did not directly affect the activity of the viral RNA polymerase complex, but seems to suppress a NS1 mediated virus supportive function. Finally, when H7N7 or H1N1v infected mice were treated with SP600125, the viral load is reduced in lungs of treated compared to untreated mice. Our data suggest that this class of ATP competitive inhibitors once optimized for antiviral action potentially represent novel drugs for antiviral intervention.

Key words: influenza, pandemic H1N1v, JNK inhibitors, MAP kinase, c-Jun N-terminal kinase

P67

Antiviral activity of some benzimidazole and spiroindolinpyrrolizidine derivatives against influenza A(H1N1) 2009 virus

Liubov A. Karpinskaya[1*], Oleg I. Kiselev[1], Anatoly S. Morkovnik[2], Liudmila N. Divaeva[2], Sergey V. Kurbatov[2], Alexandr A. Shvets[2], Vladimir V. Zarubaev[1]

[1] Influenza Research Institute, St. Petersburg, Russia
[2] Southern Federal University, Rostov-on-Don, Russia

Influenza remains a poorly controlled infection causing annual epidemics and pandemics. At the same time the number of effective anti-influenza drugs is limited and besides their wide application leads to an increase in the rate of resistant strains. This fact highlights the need to develop new tools against the flu. In recent years, numerous studies have been conducted regarding antiviral properties of substances of natural origin. From another hand the mostly used in clinical practice are compounds of synthetic origin. Particular attention should be given to derivatives of benzimidazole, because those substances have a wide spectrum of biological activity.

We have tested 22 derivatives of benzimidazole (BI) and 10 derivatives of spiroindolinpyrrolizidine, all were synthesized in Southern Federal University, Rostov-on-Don, Russia. Pandemic influenza virus A/California/7/09 (H1N1) was grown in MDCK cells in a presence of compounds. After 48 hours of incubation virus yield was evaluated in hemagglutination test. The toxicity of compounds was tested in microtetrazolium test (MTT). Based on the results obtained, the 50% cytotoxicity dose (CTD_{50}), 50% effective dose (ED_{50}) and their ratio – selective index (SI) were calculated.

The results obtained demonstrated that 7 (32%) studied BI derivatives had SI more then 10 (10-33.6) and (50%) studied compounds of spiroindolinpyrrolizidine derivatives had SI more than 10 (10.9 -29.04).

We have also shown a protective effect of the most effective BI derivative on the model of lethal influenza pneumonia in white mice. Protection index for this substance at a dose of the virus 1 LD_{50} was 75%.

In general, derivatives of BI and spiroindolinpyrrolizidine showed relatively high activity against pandemic influenza and can therefore be recommended for further development for prevention and/or treatment of influenza.

P68

Pandemic A/H1N1 (2009) Swine Influenza Virus Detection and Isolation in Commercial Piggery, Lagos Nigeria

Meseko, C.A[1, 2*], Bakarey, A.S[1], Odaibo, G.N[1] and Olaleye D.O[1]

[1, 2]WHO National Influenza Centre, Virology Department, College of Medicine, University of Ibadan, Ibadan
[1]FAO Regional Laboratory for Animal Influenza & other TADs, National Veterinary Research Institute, Vom

WHO declared the first pandemic of the 20th century in 2009 after influenza A/H1N1 of swine origin spread through 5 continents including Africa. Shortly after the outbreak waned in human, infections were reported in pigs in North America, Europe and Asia. Data on swine influenza in Africa is however lacking due to poor surveillance activity, yet knowledge of the state of influenza in this region is important for global pandemic control.
We surveyed for swine influenza virus in Nigeria at the human–animal interface in a peri-urban pig estate. Nucleic acid from swab specimens collected in virus transport medium from clinical cases was extracted with Qiagen RNAeasy commercial kit. Real time RT-PCR assay was run with extracted RNA using M and HA gene specific primers. Virus isolation was carried out on samples that were positive by molecular methods in chicken embryonated egg and identified by HI and RT-PCR subtyping.
Thirty five (16%) specimens out of 221 collected between July 2010 and June 2012 were positive for influenza A by real time RT-PCR out of which 29 (13%) were isolated and 18 (8%) identified as 2009 pandemic influenza HIN1. This is the first evidence of pandemic H1N1 swine influenza virus detection and isolation in pigs in West Africa and the second in Africa. This study underscores the importance of swine influenza surveillance at the human-animal interface in this region. Further molecular characterization would shed more light on genetic diversity and evolution of the virus.

Key words: Influenza, A/H1N1, pandemic, Pigs, Nigeria

P69

Bimolecular fluorescence complementation detects homo- and hetero-oligomerisation of Mx Proteins

Schilling Mirjam*, Haller Otto, Kochs Georg

Department of Virology, University of Freiburg, Germany

Mediating resistance to orthomyxoviruses and a broad range of other viruses, human MxA – an Interferon (IFN)-induced dynamin-like GTPase – plays a critical role in innate immunity. In contrast to MxA, there is no known antiviral activity for its homologue MxB which is simultaneously induced by IFN.
Structural and functional analysis of MxA revealed that self-assembly is important for its binding to target structures and its antiviral activity. Furthermore, oligomerisation is known to regulate GTP hydrolysis in most large GTPases.
Therefore we investigated homo- and heterooligomerisation of MxA and MxB in the absence or presence of viral infection via a bimolecular fluorescent complementation (BiFC) assay. In this assay BHK cells were transfected with expression plasmids for split-YFP tagged Mx proteins which reconstitute their fluorescence if the proteins are located in close proximity to each other.
We confirmed BiFC as a suitable tool to display specific protein-protein interactions and the oligomerisation process of Mx proteins. We detected colocalisation of oligomerising MxA and MxB proteins, as well as heteroologomers of MxA and MxB proteins.
Furthermore, we could show that viral infection leads to partial disruption of MxB oligomers and almost complete abrogation of MxA-MxB colocalisation, suggesting involvement of MxB in the IFN-induced antiviral response.
We conclude that bimolecular fluorescence complementation is a useful tool to further analyse the antiviral mechanism of Mx proteins and, in particular, the function of MxB which is not resolved until now.

Key words: keyword, keyword

P70

Model for the caspase-dependent nuclear RNP export in influenza virus-infected cells

J. Dzieciolowski[1*] , D. Mühlbauer[1], M. Hardt[2] and S. Pleschka[1]

[1] Institute of Medical Virology, Justus-Liebig-University Giessen, Schuberstr. 81, D-35392 Giessen, Germany; [2] Imaging Unit, Justus-Liebig-University Giessen, Schubertstr. 81, D-35392 Giessen, Germany

Previous work has shown that the influenza virus (IV)-induced apoptotic caspase activity promotes nuclear RNP export in the late phase of the IV replication cycle. For a better understanding of the molecular mechanisms and factors involved we investigated the effect of the caspase activity on the bottleneck of nuclear transport – the nuclear pore complex (NPC). As it is known that caspase-activity can degrade the NPC we analyzed whether this also takes place in the IV-infected cell and how this might affect NPC morphology, active cargo transport via the NPC and NPC diffusion limits. The nuclear RNP export is also CRM1-dependent and we have furthermore analyzed whether the RNP-export switches from active, CRM1-dependent transport to passive, caspase-dependent nuclear evasion. Our results so far indicate that, (i) caspase activity leads to degradation of the nuclear pore components, (ii) passive evasion of a GFP multimere tagged with a nuclear localization signal (NLS) and (iii) increased diffusion limits. Additionally, we show that impairment of virus replication by CRM1 inhibition is lost at a time point p.i. when IV-induced caspase activity increases.
The IV-induced caspase activity seems to be activated at least in part by pro-apoptotic factors such as TRAIL/Fas. Hence, we started to investigate to what extent TRAIL/FasL affect the caspase-dependent nuclear RNP export. As IV-induced apoptosis, which affects RNP export could be caused by death-ligand mediated signalling our investigation will allow correlating TRAIL/FasL-signalling with the viral replication cycle. First results will be presented.

Key words: caspase, nuclear pore complex, ribonucleoprotein complex

P71

A novel vector to clone influenza A viruses segments for the bi-directional, simultaneous generation of vRNA and mRNA using a CcdB selection marker and alternative cloning sites

Ahmed Elsayed[1*], Matthias Riedl[1] and Stephan Pleschka[1]

[1] Institute of Medical Virology, Justus-Liebig-University Giessen, Schubertstr. 81, D-35392 Giessen, Germany

Reverse genetic of influenza A virus is simply the rescue of infectious virus from transfected cDNA of the eight genomic RNA-segments of influenza A virus. For this, the viral RNA-segments are cloned as cDNA into special vectors that allow the simultaneous generation of a Pol1-driven vRNA-like transcript of the segment, as well as the Pol2-driven generation of the according mRNA leading to the expression of corresponding protein(s). One of the most critical points in cloning the viral segments into of such expression vectors is an effective selection of vectors that contain the cDNA. To this point, we constructed a bi-directional vector encoding the lethal ccdB gene as a negative selection marker flanked with two AarI restriction/cloning sites between the human Pol1-promoter and the murine Pol1-terminator based on the pHW2000 vector by Hoffmann and Webster. In E. coli, the ccd locus of the F plasmid codes for two gene products, CcdA and CcdB. The CcdB protein which has a longer half-life than CcdA interferes with the DNA-gyrase, while CcdA acts as a repressor of CcdB. Therefore expression of CcdB over CcdA leads to cell death. CcdB protein is lethal for all E. coli except the strain DB3.1 (containing gyrA462 mutation). Through this newly constructed cloning vector, we overcome the drawbacks of other available cloning vectors (pHW2000blue) encoding LacZ as a selection marker that can lead to confusing results and which contains poorly digested BsmB1 restriction sites. We established two complete reverse genetic system of A/chicken/SA/CP7/1998 (H9N2) and S-OIV A/Giessen/1/09 (H1N1) to study the impact of NS reassortment of S-OIV with HPAIV (H5-and H7-) and of LPAI (H9N2) on the S-OIV host range, replication efficiency and virus-induced cellular response.

Key words: Influenza virus, cloning vector, reassortment

P72

Small molecule inhibitors of influenza A and B viruses that act by disrupting subunit interactions of the viral polymerase

G. Muratore[1], L. Goracci[2], B. Mercorelli[1], A. Foeglein[3], P. Digard[3,4], G. Cruciani[2], G. Palù[1], and A. Loregian[1*]

[1]Department of Molecular Medicine, University of Padua, Padua, Italy; [2]Department of Chemistry, University of Perugia, Perugia, Italy; [3]Department of Pathology, University of Cambridge, Cambridge, United Kingdom; [4]The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, United Kingdom

Influenza viruses are the cause of yearly epidemics and occasional pandemics that represent a significant challenge to public health. Current control strategies are imperfect and there is an unmet need for new antiviral therapies. Here, we report the identification of the first small molecule compounds able to effectively and specifically inhibit growth of influenza A and B viruses in cultured cells through targeting an assembly interface of the viral RNA-dependent RNA polymerase. Using an existing crystal structure of the primary protein-protein interface between the PB1 and PA subunits of the influenza A virus polymerase, we conducted an *in silico* screen to identify potential small molecule inhibitors. Selected compounds were then screened for their ability to inhibit the interaction between PB1 and PA *in vitro* using an ELISA-based assay and in cells, to inhibit nuclear import of a binary PB1-PA complex as well as transcription by the full viral ribonucleoprotein complex. Two compounds emerged as effective inhibitors with IC_{50} values in the low micromolar range and negligible cytotoxicity. Of these, one compound also acted as a potent replication inhibitor of a variety of influenza A virus strains in MDCK cells, including H3N2 and H1N1 seasonal and 2009 pandemic strains. Importantly, this included an Oseltamivir resistant isolate. Furthermore, potent inhibition of influenza B viruses but not other RNA or DNA viruses was seen. Overall, these compounds provide a foundation for the development of a new generation of therapeutic agents exhibiting high specificity to influenza A and B viruses.

Key words: Influenza virus RNA polymerase, antiviral agents, protein-protein interactions

P73

Possible factors influencing the influenza vaccine induced antibody response in the elderly

B. Camilloni[1], M. Basileo[1], E. Lepri[1], A. Cherubini[2], G. Dell'Aquila[2], P. Tozzi[3], G. Bartolini [3], V. Committeri[3], A. M. Iorio[1*]

Affiliation [1]Dept Med Surg Spec and Public Health, University of Perugia; [2]IRCCS-INRCA Ancona; [3]ASL3, Umbria; Italy

Although influenza vaccination is recommended for elderly and chronically ill people, the extent of vaccine induced immune responses is variable since can be impaired by multiple factors. A different antibody response was observed in elderly people living in two nursing homes in Italy, the B-C (56) and the E-O (47) homes, after immunization with one dose of 2010/2011 trivalent MF59-adjuvanted influenza vaccine (FLUAD, NOVARTIS) (A/Perth/16/09, H3N2; A/California/7/09, H1N1; B/Brisbane/60/08) in November 2010. Vaccine immunogenicity was evaluated measuring haemagglutination inhibiting antibody titres in sera collected before and one month after immunization. No differences were found in pre-vaccination status. After vaccination a significant response was in general found against all three influenza strains both in people living in nursing home B-C and E-O. However, the immune response of people living in nursing home B-C satisfied all the requirements of the European Commission for elderly people whereas the required values were not reached in many instances in the elderly living in nursing home E-O. The possible factors influencing the different response were evaluated examining the health status of the elderly people using data collected in the Italian VAOR (Valutazione Anziano Ospite di Residenza) schedule (a multiple-dimensional valutation protocol). Significant differences were found for bedridden, coma, chronic cardiac ischemia, hypertension, inappropriate behavior and use of restraints. Moreover comparing people on the basis of the presence or not of protective antibody titers (≥40) after vaccination, significant differences were found for bedridden, bipolar disorders and use of restraints.

Key words: influenza vaccine, institutionalized elderly, antibody response

P74

Studies of the host response to influenza A virus infections in mouse knock-out mutants

Mohamed A. Tantawy [1,2*], Bastian Hatsuer [1], and Klaus Schughart

[1] Helmholtz Centre for Infection Research, Braunschweig, Germany; [2] National Research Centre, Giza, Egypt.

The genetics of the host represents an important factor in the severity of an infection with Influenza A virus (IAV). However, in humans genetic heterogeneity and other factors can all make the analysis of genetic very difficult. The mouse model of IAV infection has been useful in dissecting influenza pathogenesis. Importantly, genes originally found in mice were shown to play an important role in human susceptibility to IAV infection, providing evidence for the conservation of host immune defense. The objective of this work is to determine the contribution of specific host genes for the defense against IAV infections. For this, the role of the interferon alpha-inducible protein 27 like 2A (*Ifi27l2a*) gene was analyzed in mouse knock-out (KO) mutants. *Ifi27l2a* is a small hydrophobic protein localized in mitochondria and inhibiting adipocyte differentiation and mitochondrial biogenesis. The expression analysis of *Ifi27l2a* in non-infected mice by reverse transcription PCR showed that gene was expressed in lungs, spleen, salivary gland, liver and kidney. Furthermore, the β-galactosidase reporter gene which was under the control of the *Ifi27l2a* locus was highly expressed in non-infected mouse lungs. Also, *Ifi27l2a* was highly up-regulated in mice after infection with IAV. However, after challenging with either PR8 (H1N1) or SC35M (H7N7) IAV, KO mice did not exhibit a significant phenotype compared to wild type mice. Presently, in vitro primary macrophage cell culture assay are being tested to study a potential effect of the *Ifi27l2a* gene for macrophage functions.

Key words: Influenza A viruses, interferon alpha-inducible protein 27 like 2A gene, knock-out mice

P75

EPs® 7630 (Umckaloabo®), an extract from *Pelargonium sidoides* roots, exerts anti-influenza virus activity *in vitro* and *in vivo*

Linda L. Theisen[1*] and Claude P. Muller [1]

[1] Institute of Immunology, Centre de Recherche Public de la Santé/ Laboratoire National de Santé, 20A rue Auguste Lumière, L-1950 Luxembourg, Luxembourg

A prodelphinidin-rich extract from *Pelargonium sidoides* DC, EPs® 7630 (Umckaloabo®), which is licensed to treat respiratory tract infections such as acute bronchitis, was investigated for its antiviral effects. EPs® 7630 showed dose-dependent anti-influenza activity at non toxic concentrations against pandemic H1N1, Oseltamivir sensitive and resistant seasonal H1N1, seasonal H3N2 and the laboratory H1N1 strain A/Puerto Rico/8/34, while it had no antiviral activity against adenovirus or measles virus. The extract inhibited an early step of influenza infection and impaired viral hemagglutination as well as neuraminidase activity.However, EPs® 7630 did not exhibit a direct virucidal effect, as virus preincubation (unlike cell preincubation) with the extract did not influence infectivity. Importantly, EPs® 7630 showed no propensity to resistance development *in vitro*. Analysis of EPs® 7630 constituents revealed that prodelphinidins represent the active principle. Chain length influenced antiviral activity, as monomers and dimers were less effective than oligo- and polymers. Importantly, gallocatechin and its stereoisomer epigallocatechin exert antiviral activity also in their monomeric form. In addition,EPs® 7630 administered by inhalation significantly improved survival, body weight and body temperature of influenza-infected mice, without obvious toxicity, demonstrating the benefit of EPs® 7630 in treatment of influenza.

Key words: Pelargonium sidoides, polyphenol, gallocatechin, antiviral, prodelphinidin

P76

Antiviral activity of *Enterococcus faecium* on the replication of Swine Influenza Virus *in Vitro*

Zhenya Wang[1*], Michael Burwinkel[2], Michael F. G. Schmidt[3]

Probiotic microorganisms and other health improving food supplements have been given increasing attention in recent years. But information on the effect of probiotics on swine influenza viruses (SwIV) is not yet available. Here we address this question by assessing the inhibitory potential of the probiotic *Enterococcus faecium* NCIMB10415 on the replication of two porcine strains of influenza virus (H1N1 and H3N2 strain) in a continuous porcine macrophage cell line (3D4/21) derived from lung macrophages and in the continuous epithelial cell line, MDBK cells. Cell cultures were treated with *E. faecium* at the non-toxic concentration of $1x10^6$ CFU/ml in growth medium for up to 90 min before, during and after SwIV infection. After further incubation of infected cultures in probiotic-free growth medium, cell viability and virus propagation were determined two or four days later. The results show that *E. faecium* protects cell viability and inhibits virus yields by up to four log units. In both 3D4- and MDBK-cells *E. faecium* stimulated NO release which supports published evidence for an antiviral function of NO. Furthermore, this probiotic caused a modified cellular expression of selected mediators of defence in 3D4-cells: while the expression of TNF-α, TLR-3 and IL-6 were decreased in the SwIV-infected and probiotic treated cells, IL-10 and IFN-α were found to be increased. Since we recorded adsorptive trapping of SwIV through *E. faecium*, this probiotic acts at least by two mechanisms, direct physical interaction with virus particles and modulation of innate defence of the host cells.

Key words: probiotic, swine influenza viruses (SwIV), cell viability, cytokines, nitric oxide

P77

Activity of synthetic peptide glutamyl-tryptophane in combination with glycirrhizinic acid against lethal influenza on mouse model

Vladimir V. Zarubaev[1*], Angelica V. Garshinina[1], Nelly A. Kalinina[1], Anna A. Shtro[1], Liubov A. Karpinskaya[1], Pavel M. Anfimov[1], Svetlana V. Beliaevskaya[1], Viacheslav S. Smirnov[2]

[1] Influenza Research Institute, St. Petersburg, Russia
[2] CytoNIR, Ltd, St. Petersburg, Russia

Background. Due to high frequency of mutation and rapid evolution, influenza remains a poorly controlled infection. Outbreaks of highly pathogenic H5N1 influenza virus infection and appearance in 2009 of a new pandemic influenza A virus have triggered renewed interest in influenza. In order to achieve wide range of antiviral activity of the drugs, cellular pro- or antiviral pathways can be inhibited or stimulated, correspondingly. We studied here the activity of glutamyl-tryptophane (Glu-Trp), a low- molecular synthetic dipeptide, and natural triterpene glycirrhizinic acid (GA), alone or in combination, against influenza virus.

Materials and methods. Mice were infected with mouse-adapted influenza viruses strain A/Aichi/2/68 (H3N2) or mouse- adapted A/California/07/09 (H1N1)pdm09, treated with Glu-Trp/GA starting 24 hours p.i. and observed for two weeks for mortality and weight loss. Infectious activity of the virus in lung tissue was determined by titration in MDCK cells and structure of the lungs was studied by morphology and morphometric analysis.

Results. We showed that Glu-Trp and GA possess high efficacy in protection of animals against lethal infection caused by both influenza viruses. In particular, the combination of these compounds reduced mortality of infected animals up to 79%, suppressed the virus' replication in the lung tissue (to 3 logs) and normalized weight dynamics comparing to placebo- treated group. Its application also led to normalization of morphology of lung tissue both on acute and chronic stage of influenza, restriction of foci of inflammation, degree of infiltration and decreasing the tissue edema and damage. Glu-Trp/GA combination strongly affected the composition of cells of bronchial epithelium increasing the rate of morphologically intact cells (77 vs. 34%) and decreasing that of virus-damaged and dead epitheliocytes (6 vs. 50% in Glu-Trp/GA - and placebo- treated group, correspondingly) thus preventing the increasing of permeability of basal membrane. The protective properties of the studied combination were comparable with those of reference compound Tamiflu.

Conclusion. Due to activity of Glu-Trp/GA this combination should be considered as prospective tool for treatment of influenza, in particular of severe cases, and further developed for including into complex treatment of influenza infection.

P78

Identification of Interferon stimulated genes in the chicken

Susanne Röll[1*], Carsten Krohmann[1], Peter Staeheli[2], Bernd Kapsers[1], Sonja Härtle[1]

[1] Institute for Animal Physiology, Department for Veterinary Sciences, LMU Munich, Germany; [2] Department of Virology, University of Freiburg, Germany

As in mammals type I interferons (IFN) are an essential part of the antiviral immune response in the chicken. But while in mice Mx proteins are the key effector molecules during the immune response against influenza viruses, the chicken's Mx protein is induced by IFN but does not mediate antiviral activity. Hence the aim of this study was to identify chicken Interferon stimulated genes (ISGs).
Therefore we combined an extensive comparative database analysis to identify chicken homologues to mammalian ISGs and a transcriptome analysis of spleen and lung at different time points after i.v. injection of recombinant IFN. Array analysis detected for both tissues at all time points massive changes in the transcriptome. Since only 25% of the regulated genes in the lung and even less of the regulated genes in the spleen were in agreement with the chicken ISGs from databases, this *in vivo* experiment led to the identification of a multitude of so far unknown chicken ISGs.
To test these results in different infection models, further microarray studies were performed on lungs and spleens from chicken infected with New Castle Disease (NDV) or FluAV (H5N1/R65). These investigations confirmed a large part of the newly identified ISGs. In all experiments a rapid and massive induction of IL-22 and IL-6 was detectable as well as a strong upregulation of the chemokines CCL19 and K203 (a chicken MIP family member). Another highly induced gene was the chicken homologue for IFIT-5, suggesting a conserved antiviral activity of the IFIT gene familiy.

P79

Shift of oxygen saturation in the blood of different mouse inbred strains after influenza A infection

N. Mehnert[1*], B. Hatesuer[1], K. Schughart[1]

[1]Department of Infection Genetics, Helmholtz Centre for Infection Research and University of Veterinary Medicine Hannover, 38124 Braunschweig, Germany

The course of disease after influenza A infection depends, in addition to the virulence of the virus, strongly on the predisposition of the host. So far, our studies on different mouse laboratory inbred strains revealed a strong influence of the genetic background to the susceptibility of the host. The host-specific immune response was analyzed on the basis of the susceptible DBA/2J and the more resistant C57BL/6J strains. However, the exact causes of the high susceptibility exhibited by the DBA/2J mice are not fully understood. In this work, the different phenotypes of these mouse strains were investigated by means of pulse oximetry.

We established pulse oximetry as an additional measurement to body weight loss to better follow the physiopathology after influenza A virus infection. The results showed a decrease in lung function of different inbred mouse strains during the course of infection. However, during the first days post infection the oxygen saturation was still high and decreased about three days later than the body weight loss was evident. Regeneration of oxygen saturation started after clearance of the virus about two days later than regeneration of the body weight. Whereas body weight loss was more dramatic in mice after administration of virus high doses or of highly virulent virus, the reduction in blood oxygen saturation did not increase accordingly. In conclusion, the measurement of blood oxygen saturation by pulse oximetry in mice reflects pathophysiological changes after influenza A virus infection but does not provide a robust measurement for the severity of disease.

Key words: Influenza A, mouse, pulse oximetry, physiological phenotyping

P80

Introduction of silent mutations into the NP gene of influenza A viruses as a possible strategy for the creation of a live attenuated vaccine.

D. Anhlan[1*], E. R. Hrincius [1], C. Scholtissek [2], S. Ludwig [1]

[1]Institute of Molecular Virology (IMV), University of Muenster, Von-Esmarch Strasse 56, D-48149 Muenster, Germany;
[2]Waldstrasse Strasse 53, D-35440 Linden, Germany

The nucleoprotein (NP) of influenza A virus (IAV) is associated with many different functions including host range restriction. Multiple sequence alignment analyses of 748 NP gene sequences from GenBank revealed a highly conserved region of 60 nucleotides within the ORF at the 3´ends of the cRNA, in some codons even silent mutations were not found. This suggests that the RNA structure integrity within this region is crucial for IAV replication. To explore the impact of these conserved nucleotides for viral replication we created mutant viruses with one or more silent mutations in the respective region of the NP gene of the IAV strain A/WSN/33 (H1N1) (WSN). Assessment of viral replication of these WSN mutant viruses showed significant growth disadvantages when compared to the corresponding parental strain. On the basis of these findings we tested whether the attenuation of IAV by introduction of silent mutations into the NP gene may serve as a strategy to create a live attenuated vaccine. Mice vaccinated with the attenuated WSN mutant survived a lethal challenge dose of wild type WSN virus or the mouse adapted pandemic H1N1v strain A/Hamburg/4/2009. Thus, introduction of silent mutations in the NP of IAV is a feasible approach for a novel vaccination strategy allowing attenuation of the master strain but leaves the antigenicity of the gene product unaltered. This principle is potentially applicable for all viruses with segmented genomes.

Key words: Live attenuated vaccine, Influenza A virus, Nucleoprotein (NP) gene, Silent mutation, Cross-protective immunity

P81

Shedding of resistant and susceptible influenza viruses in infants during oseltamivir treatment

B.Rath[2], M. von Kleist[3], F. Tief[2], K. Karsch[2], E. Tuerk[2], S. Muehlhans[2], F. Louis[4], H. Skopnik[4], B. Schweiger[1], S. Duwe[1*]

[1]Robert Koch-Institut, Division of Influenza/Respiratory Viruses, National Reference Centre for Influenza, Berlin; [2]Department of Pediatrics, Division of Pneumology-Immunology, Charité University Medical Center Berlin; [3]Computational Pharmacometrics Group, Department of Mathematics and Computer Science, Free University Berlin; [4]Department of Pediatrics and Adolescent Medicine, Klinikum Worms.

In Germany, only the neuraminidase inhibitors oseltamivir and zanamivir are currently available for therapy and prophylaxis of influenza A(H1N1)pdm09 and influenza B infections. The orally administered oseltamivir is used as a first-line treatment. Resistance due to the H275Y NA-substitution has been reported, but little is known about viral shedding and resistance development during treatment in infants infected with influenza A(H1N1)pdm09 and influenza B viruses.

Laboratory-confirmed and oseltamivir-treated cases of A(H1N1)pdm09 (n=27) and influenza B (n=9) virus infections in infants were examined at baseline, at day five and in additional follow-up samples, if available. Viral shedding profiles were determined by quantitative RT-PCR. Resistance analysis has been carried out by using classical sequencing of original nasopharyngeal specimens and additionally, in cases of positive viral culture, fluorometric neuraminidase inhibition assays.

All 36 examined influenza infections were susceptible to both neuraminidase inhibitors at baseline prior to oseltamivir therapy. In the group of A(H1N1)pdm09 infected patients, seven out of twenty-seven (26%) developed the neuraminidase H275Y substitution conferring oseltamivir resistance. The median duration of viral shedding was significantly longer in drug resistant compared to sensitive A(H1N1)pdm09 and influenza B infections. However, once clearance was achieved, no viral load rebound occurred, suggesting a self-limiting infection even with resistant viruses.

Our data indicate a rapid development of resistant A(H1N1)pdm09 viruses and an extended shed of such viruses during therapy. Monitoring of shedding and resistance profiles may help to optimize duration and regime of antiviral treatment.

Key words: resistance, shedding, oseltamivir, infants

P82

Growth Of Porcine Influenza Viruses In Differentiated Respiratory Epithelial Cells

A. Fandan Meng[1]*, B. Darsaniya Punyadarsaniya[2], C. Xiaofeng Ren[3], D. Georg Herrler[1]

[1]Institute of Virology, University of Veterinary Medicine, Hannover, Germany; [2]Institute of Virology , Mahanakorn University of Technology, Bangkok, Thailand; [3]College of Veterinary Medicine, Northeast Agricultural University, Harbin, China.

We have recently reported precision-cut lung slices (PCLS) as a model system to analyze the infection of porcine influenza viruses in their natural target cells (Punaydarsaniya et al., PlosOne 6:e28429, 2011). Comparison of a porcine virus of the H3N2 subtype with an avian virus of the H9N2 subtype revealed differences in the virulence as indicated by various parameters: (i) duration of the growth cycle, (ii) amount of infectious virus released into the supernatant, and (iii) extent of the ciliostatic effect. Here we compared three porcine viruses of the three subtypes currently prevalent in the swine populations (H3N2, H1N1, H1N2). Comparison of the data with pathogenicity data in pigs and mice reveals which of the above parameters is the best indicator of the pathogenicity of porcine influenza viruses.

Key words: respiratory epithelium, precision-cut lung slices, porcine influenza viruses

P83

Experimental infection of Common gull (Larus canus) with H5N1 highly pathogenic avian influenza virus

E. Sherstkov[1,2*], A. Zaykovskaya[2], A. Shestopalov[2], L. Shestopalova[1]
[1]Novosibirsk State University; [2]Federal State Research Institution State Research Centre of Virology and Biotechnology "VECTOR", Novosibirsk, Russia

The present study investigated the susceptibility of Common gull (Larus canus) exposed to H5N1 highly pathogenic avian influenza (HPAI) virus. We infected twenty-eight 6-weeks-old gulls applied to the nares, conjunctiva and throat with 10^7 median embryo infectious doses of A/Common gull/Chany/2006 (H5N1) (IVPI=1,7). This virus was isolated from a clinically healthy gull in Russia in 2006.
We observed infected gulls for 28 days. Every day we collected throat and cloacal swabs; samples of viscera – in 2, 4, 5, 6, 8, 10, 14, 18, 21 and 28 days after contamination for histological and virological studies.
During observation period we recorded diarrhea, decrease of appetite and motion activity, hyperemia of pharynx, necrotic lesions of legs, torticollis. Influenza viruses were detected in throat and cloacal swabs in 1–25 days after inoculation by used rRT-PCR and virus isolation on embryo eggs. Also HPAI was found in tissues of brain, air sacs, lungs, trachea, liver, heart, large intestine, kidney, spleen, bursa of Fabricius, esophagus and stomach. Major histological lesions were acute meningoencephalitis, acute myocarditis, acute pancreatic necrosis, multifocal necrotizing hepatitis, interstitial pneumonia, destruction of respiratory epithelium, small-focal necrosis in lungs. Mortality were observed in the gulls in 5-18 days after inoculation (7 of 28 infected gulls).
This shows that the Influenza virus A/Common gull/Chany/2006 (H5N1) provokes serious lesions of viscera in Common gull that could resulted in fatal outcome; however the surviving gulls are able to excrete the HPAI until 25 days after infection.
This work was supported by FAP State contract grant №16.740.11.0179.

Key words: Common gull, experimental infection, HPAI

P84

Structure of humoral immune response to the pandemic influenza A(H1N1)pdm09 virus in vaccinated and unvaccinated patients

A.A.Vasilyeva[1]*, E.M.Voitsehovskaya[1], V.S.Vakin[1], E.V.Kuznetsova[1], Y.A.Dagil[2], V.Z. Krivitskaya[1], A.A.Sominina[1]

[1]Research Institute of Influenza, Saint-Petersburg, Russia
[2]"Petrovax Pharm", Moscow, Russia

Structure of humoral immune response to seasonal influenza A(H1N1) and A(H1N1)pdm09 viruses was investigated in 38 adult patients with pandemic influenza in 2011. Part of the patients (n=21) was vaccinated by trivalent inactivated vaccine to A(H1N1)pdm09, A(H3N2) and B influenza viruses 3 month before disease.
Antibodies specific to A/Brisbane/59/07 (H1N1) and A/California/07/09 (H1N1)pdm09 influenza viruses were estimated in paired sera by microneutralization assay (MN). Besides, immunoglobulin isotypes IgG1, IgG2, IgG3 were analyzed in ELISA using purified surface glycoproteins of indicated above viruses.
The level (OD_{450}) of A/California/07/09-specific IgG1 in ELISA in the first 3 days of disease was higher in group of vaccinated as compared with unvaccinated one (0,595 and 0,311 respectively). A(H1N1)pdm09-specific neutralizing antibody geometric mean titers (GMT) in sera obtained in acute phase were estimated as 1:30 in the group of vaccinated and 1:8in nonvaccinated one. Thus, GMTwere 3,7 times higher in vaccinated patients as well.
The high A/California/07/09-specific IgG1conversion rate was observed in both vaccinated and unvaccinated patients (70,5-76%). Seroconversion rates of (H1N1)pdm09-specific IgG3 and especially IgG2 were low in both groups (0-29%). Virus neutralizing antibodiesamong the patients were registered in 60% of cases in the group of vaccinated and 64% of cases in the group of nonvaccinated.
Rate of cross-reacting antibodies registration in ELISA simultaneouslyto A/California/07/09 and A/Brisbane/59/07 viruses in vaccinated patients was higherthan in unvaccinated group (48 and 23,5% respectively).
Thus, preliminary results indicated that antibody GMT in MN estimated as 1:30 didn`t protect from natural infection associated with A/California/07/09-like influenza viruses.

Key words: pandemic influenza A(H1N1)pdm09 virus, antibody isotypes, inactivated vaccine

P85

Discrimination between α2-3- and α2-6-sialylated receptor gangliosides of influenza A virus using immunodetection combined with electrospray ionization mass spectrometry

G. Pohlentz[1*], I. Meisen[1], M. Mormann[2], H. Karch[1], J. Müthing[1]

[1]Institute for Hygiene, University of Münster, Münster, Germany; [2]Institute of Medical Physics and Biophysics, University of Münster, Münster, Germany

Gangliosides are a variable class of glycosphingolipids (GSLs) composed of a ceramide portion (*N*-acylsphingosine) as lipid anchor, and an oligosaccharide moiety containing one or more sialic acid residues. Gangliosides with terminal Neu5Acα2-3Galβ1-4GlcNAc- or Neu5Acα2-6Galβ1-4GlcNAc-sequences represent potential binding sites for influenza A viruses. The thin-layer chromatography (TLC) overlay technique is an appropriate method to discriminate between these differently sialylated gangliosides using specific antibodies. Mass spectrometry (MS) allows for elucidating the monosaccharide sequence of the glycan core and, in particular, the linkage position of the sialic acid as well as the structure of the ceramide moiety of individual ganglioside species.
In the present study we matched three complementary methods comprising (1) TLC separation of ganglioside mixtures, (2) ganglioside detection with sialyloligosaccharide-specific antibodies, and (3) electrospray ionization (ESI) MS analysis of gangliosides after extraction from the silica gel of immunopositive bands. A clear-cut series of fragment ions with discriminatory significance was obtained for both types of isomeric gangliosides carrying α2-3- and α2-6-linked Neu5Ac, respectively, by use of collision induced dissociation MS. A characteristic sialic acid ring cleavage was detected exclusively in all species with Neu5Acα2-6Galβ1-4GlcNAc terminus, regardless of the chain length of the oligosaccharide core and ceramide fatty acid heterogeneity, leading to the unambiguous discrimination of α2-3- and α2-6-sialylated neolacto-series monosialogangliosides. The combined method works on submicrogram scale of ganglioside mixtures and offers a promising tool for screening of specific ganglioside virus receptors in small human or animal tissue samples and may thus be helpful to explain the poorly understood virus organ tropism.

Key words: gangliosides, sialic acid, TLC, mass spectrometry

P86

Detection of antiviral-resistant influenza A(H1N1)pdm09 viruses in Japan by a combination of chemiluminescent and fluorescent neuraminidase inhibitor susceptibility assays

E. Takashita[1*], M. Ejima[1], S. Fujisaki[1], N. Kishida[1], H. Xu[1], M. Imai[1], N. Kim[1], A. Sato[1], H. Sugawara[1], R. Itoh[1], T. Doi[1], M. Tashiro[1], T. Odagiri[1]

[1]Influenza Virus Research Center, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan

In Japan, four neuraminidase inhibitors (NAIs), oseltamivir, peramivir, zanamivir, and laninamivir, are approved and prescribed in clinics for chemotherapy against influenza with the highest frequency in the world. Consequently, sustained monitoring of antiviral drug-resistant viruses in national level is important in Japan. We have monitored 11,963 A(H1N1)pdm09 viruses since May 2009 by both allele-specific RT-PCR assay to detect H275Y mutation in NA gene and susceptibility assays to NAIs. As of May 2012, we detected 157 (1.3%) oseltamivir- and peramivir-resistant viruses in clinical isolates and observed that the percentage of H275Y resistant viruses from the cases with no known exposure to NAIs increased from 20% during the 2008-2009 season to 44% during the 2010-2011 season. A number of person-to-person transmission cases were confirmed, although the resistant viruses did not spread in community. Out of 157 resistant viruses, 28 (18%) were mixed isolates of 275H and 275Y (275H/Y) viruses with a minor composition of the H275Y mutation. By chemiluminescent (CL) assay, they exhibited indistinguishable susceptibility to oseltamivir and peramivir from the sensitive isolates with 275H, whereas by fluorescent (FL) assay, they were clearly discriminated between 275H/Y and 275H isolates. From our results, it is recommended that a combination of CL and FL assay systems should be used in antiviral-resistant surveillance to precisely evaluate susceptibility to NAIs.
This study was carried out by collaboration with the influenza virus surveillance group of Japan.

Key words: antiviral, resistance, neuraminidase inhibitor

P87

Induction of Apoptosis by Seasonal and Highly Pathogenic IAV in *ex vivo* infected Human Lung Tissue

A. Becher[1*], V.K. Weinheimer[2], T. Bauer[3], M. Tönnies[3], A. D. Gruber[4], T. Wolff[2], N. Suttorp[1], S. Hippenstiel[1,] A. Hocke[1]

[1] Department of Internal Medicine/Infectious Diseases and Pulmonary Medicine, Charité – Universitätsmedizin Berlin, Germany; [2] Div. of Influenza/Respiratory Viruses, Robert Koch Institute, Berlin, Germany; [3] HELIOS Klinikum Emil von Behring, Berlin, Germany; [4] Department of Veterinary Pathology, College of Veterinary Medicine, Freie Universität Berlin, Germany

Unlike seasonal influenza A viruses (IAV), highly pathogenic IAV of the subtype H5N1 infect the lower respiratory tract of humans and consequently cause viral pneumonia rapidly progressing to ARDS (acute respiratory distress syndrome) and respiratory failure. Autopsies of fatal H5N1 cases have shown diffuse alveolar damage (DAD). IAV have been shown to induce apoptotic cell death both *in vivo* and *in vitro*. Since type II alveolar cells are the major target cells of IAV, apoptosis of these cells may play a critical role in development of DAD and therewith respiratory failure.
Ex vivo infected human lung tissue was used to compare apoptosis in alveolar epithelial cells induced by a seasonal virus of the subtype H3N2 and a highly pathogenic virus of the subtype H5N1. An immunohistochemical approach in combination with digital image analysis showed an increase in cleaved caspase 3 positive cells in infected areas for the seasonal and the highly pathogenic IAV compared to non-infected areas 48h post infection. Both viruses showed no differences regarding the rate of induced apoptosis, implying that their differential pathogenicity can not be explained by apoptotic cell death in this model. Interestingly, only a very small amount of IVA-positive cells were apoptotic. The increase of apoptotic cells in the infected areas, which mostly accounts for non-infected cells, suggests that the type I interferon response of infected type II alveolar cells creates an antiviral state in the surrounding tissue by induction of IFN stimulated genes (ISGs), of which several have been identified with apoptotic function.

Key words: Influenza A, human lung, apoptosis

P88

The significance of the influenza, ARI and SARI surveillance system in Republic of Moldova

C.Spînu[1], P.Scoferţa[1], R.Cojocaru[1*], V.Eder[1], C.Rusu[1], I.Spînu[1], S.Gheorghita[1]
[1]National Center for Public Health, Ministry of Health, Republic of Moldova

Surveillance of influenza, acute respiratory infections (ARI) and severe acute respiratory infection (SARI) in Moldova is made according to criteria recommended by EuroFlu Surveillance System, such as: geographical spread, intensity and trend of the epidemic process, impact on health services, dominant type of influenza virus.
During the epidemic season w40/2011-18/2012 in Moldova there were sporadic cases of influenza, caused mainly by influenza A(H3N2), with a low intensity of the epidemic process and minimal impact on medical services. During this season were registered 227 ($637^0/_{0000}$) influenza cases.
ARI morbidity ranged from $50.4^{0/}{}_{0000}$ (w52/2011) to $179.3^0/_{0000}$ (w11/2012), falling below the epidemic threshold ($187.0^0/_{0000}$), peaking ($201.9^0/_{0000}$) at w12/2012. This season were registered 139 964 ($3929.6^0/_{0000}$) ARI cases.
SARI morbidity ranged from $10.8^0/_{0000}$ (w40/2011) to $48.3^0/_{0000}$ (w3/2012) subsequently decreases successively and continuously until the end of the season. During this time had been recorded 36,932 ($1036.9^0/_{0000}$) cases of SARI. Ascending trend values (3.6) and downward (3.4) of influenza morbidity, ARI and SARI during the period of surveillance practically are without difference.
Application of influenza, ARI and SARI surveillance system connected to the WHO, ECDC requirements has allowed us to monitor the epidemiological situation in these infections, appreciation of the epidemic process trend and the spread forecast with developing control and response measures. The study of influenza virus show prevalence of dominant virus A(H3N2) - 95.7% for the 2011-2012 season and 58.9% of A(H1N1)pdm09 - for the 2010-2011 season.
SARI morbidity served as an argument for extending the range of population on increased risk quotas for influenza immunization for the season 2012-2013 with influenza vaccine recommended by WHO.

Key words: influenza, surveillance, ARI, SARI

P89

The role of the FGF10/FGFR2b axis in epithelial repair following severe influenza pneumonia

J. Quantius[1*], O.R. Gottschald[1], J. Lohmeyer[1], S. Herold[1]

Department of Internal Medicine II, Justus-Liebig-University, Giessen, Germany

Influenza virus (IV) pneumonia is associated with apoptotic damage of the alveolar epithelial barrier and pulmonary edema, therefore efficient alveolar repair is crucial for recovery. Cell lineage tracing studies suggest that the adult lung contains epithelial progenitor cells which are resistant to damage and proliferate after injury. FGF10 plays a major role in lung development, but it is also known to have reparative and anti-apoptotic potential after lung injury.

Analysis of CD31- CD45- lung cells according to established surface marker expression revealed different epithelial populations with proliferative potential one of which is defined as $EpCAM^{high}$ and $CD24^{low}$. During influenza virus infection this putative progenitor population showed high IV infection rates but partial resistance to apoptosis. The FGF10 receptor FGFR2b was significantly upregulated on these progenitor cells on day 5 post IV infection compared to naphthalene treatment and comprises the highest surface expression in the acute phase of IV pneumonia. Non-infected progenitor cells show significant FGFR2b upregulation and higher proliferation rates compared to infected progenitor cells. Of note, mesenchymal cells, the primary source of FGF10 during lung development and after injury, were found to be IV infected at low amounts. Intratracheal application of FGF10 in the acute phase, during FGFR2b upregulation, significantly increased the reparative response of putative epithelial progenitor cells.

Identification of the molecular pathways involved in the reparative signaling events after IV-induced lung injury and the role of epithelial-mesenchymal cross-talk may result in new potential targets for intervention to attenuate severe IV-induced lung injury and drive tissue repair.

Key words: Lung injury, progenitor cells, alveolar repair, FGF10

P90

Components of the human interactome of influenza A virus ribonucleoproteins revealed by RNA tagging and proteomics

A. York[1*], E. Fodor[1]

[1]Sir William Dunn School of Pathology, University of Oxford

Influenza A viruses encode for a limited number of proteins and are therefore highly reliant on numerous host cellular functions to support their replication cycles. Identifying cellular interaction partners of the virus is critical for a comprehensive understanding of the life-cycle of influenza A viruses. Here we report an integrative RNA tagging and proteomic approach being employed to identify cellular factors that interact with viral ribonucleoproteins during infection.

Genomic single-stranded viral RNA (vRNA) is replicated via a complementary (cRNA) intermediate, both of which are encapsidated in viral nucleoprotein and bound by the heterotrimeric RNA-dependent RNA polymerase to form viral ribonucleoprotein (vRNP) and complementary viral ribonucleoprotein (cRNP) complexes. The heterotrimeric viral polymerase is also responsible for viral mRNA synthesis via a cap-snatching mechanism.

Recombinant influenza A/WSN/33 viruses have been generated by reverse genetics to contain an RNA tag which is bound by the *Pseudomonas aeruginosa* phage 7 coat protein. In combination with a recently described Strep-tagged PB2 subunit, these tags have been exploited for the affinity purification of influenza A vRNPs and cRNPs from infected cells with high specificity. Identification of cellular factors co-purifying with viral RNPs by liquid chromatography-tandem mass spectrometry has revealed a number of candidate proteins that interact directly with the virus, which may us help to understand the role of the host cellular machinery during influenza A virus infection.

Key words: Influenza, Host-pathogen interactions, RNA-affinity purification, Strep purification, LC-MS/MS

P91

Contrasting regulation of suppressors of cytokine signalling (SOCS) between human and pig tracheal epithelial cells following highly pathogenic avian influenza virus infection

A. Chang Pengxiang[1*], B. Suresh V Kuchipudi[1], C. Rahul Nelli[1], D. Kin-Chow Chang [1]

[1,] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, College Road, Loughborough, Leicestershire LE12 5RD, UK

Human cases of highly pathogenic avian influenza A (HPAI) H5N1 infections frequently result in severe to fatal lung and systemic damage, which is ascribed to the excessive induction of pro-inflammatory cytokines, a condition known as hypercytokinemia or cytokine storm. By contrast, HPAI H5N1 infection in pigs produces mild to no clinical effects. Members of the suppressor of cytokine signalling (SOCS) family (SOCS1 to 7 and CISH), have been identified as crucial negative regulators of cytokines that signal through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway during many inflammatory conditions. We hypothesise that members of the suppressor of cytokine signalling (SOCS) gene family play crucial moderating roles in the pathogenesis of hyperacute inflammatory response to HPAI H5N1 infection.

Here, we adopted a comparative approach to examine the regulation of SOCS genes in vulnerable human and resistant pig tracheal epithelial cells (TECs) in response to moderate and high pathogenicity influenza viruses. Human and pig TECs infected with a moderately pathogenic human H1N1 virus (A/USSR/77) showed a similar transcriptional up-regulation of most members of the SOCS gene family. Interestingly, we found that most members of the SOCS family were transcriptionally down-regulated in human TECs but up-regulated in pig TECs infected with HPAI H5N1 virus (A/turkey/Turkey/1/05). Our data suggest a possible pivotal role played by members of the SOCS family in the regulation of host pro-inflammatory response and the pathogenesis of HPAI H5N1 virus infection in humans and pigs.

Key words: Human, pig, influenza, SOCS

P92

Phosphatidylinositol-3-kinase (PI3K) is activated by influenza virus vRNA via the pathogen pattern receptorRig-I to promote efficient type I interferon production

Rüdiger Dierkes*, Eike R. Hrincius, Darisuren Anhlan, Viktor Wixler, Stephan Ludwig, and Christina Ehrhardt

Institute of Molecular Virology (IMV), Centre of Molecular Biology of Inflammation (ZMBE) Westfälische-Wilhelms-University, Von Esmarch-Str. 56, D-48149 Muenster, Germany

The phosphatidylinositol 3-kinase (PI3K) is activated upon influenza A virus infection in a biphasic manner. An early and transient induction of PI3K signaling is induced by viral attachment to cells and promotes virus entry. In later phases of the infection cycle the kinase is activated by direct interaction with the viral NS1 protein leading to prevention of premature apoptosis induction. Besides these virus-supporting functions, it was also suggested that PI3K signaling is essential for full activation of interferon regulatory factor 3 (IRF-3) in response to synthetic dsRNA and IAV infections. However, a direct role of PI3K signaling in the innate immune response to influenza virus infections was not described yet. Here we show that accumulation of vRNA in human lung epithelial cells infected with either influenza A or B viruses results in PI3K activation. Furthermore, expression of the RNA receptors Rig-I and MDA5 was increased upon stimulation with virion-extracted vRNA or IAV infection. Using siRNA approaches, Rig-I was identified as the pathogen receptor necessary for influenza virus vRNA sensing and subsequent PI3K activation in a TRIM25 and MAVS signaling dependent fashion. Rig-I induced PI3K signaling was further shown to be essential for full IRF-3 activation resulting in the induction of the type I interferon response. These data show that PI3K is activated as part of the Rig-I mediated anti-pathogen response to enhance expression of type I interferons.

Key words: PI3K, Rig-I, IFN response, vRNA

P93

Comparison of data of Influenza Routine vs. Sentinel Surveillance System in Georgia, influenza seasons 2009-2010/2010-2011

O. Tarkhan-Mouravi[1*], Kh. Zakhashvili[2], I. Karseladze[3], G. Kandelaki[4]

[1] National Center for Disease Control and Public Health of Georgia

Since 2012 influenza surveillance system transited from routine with monthly aggregated data on Acute Respiratory Illness (ARI)/ Influenza and sentinel to only sentinel ILI/SARI surveillance. In order to prove the decision the profits of both systems were compared.
Routine and sentinel surveillance data of influenza seasons 2009-2010/2010-2011 in Georgia was analysed. Country population for routine surveillance and catchment population of sentinel sites, weekly visits/admittions were used as denominators for incidence rates.
By routine surveillance data (RSD), influenza incidence (per 100,000) during season 2009-2010 was unusually high due to pandemic with pick in December (592.9) and continued up to March (72.5) in 2010 and by sentinel surveillance data (SSD) season ILI peak was on week 51st (591.0); only pandemic H1N1 circulated. As to season 2010-2011 incidence pick in February (429.9) by RSD and on weeks 5th (1995.3) by SSD, was caused by simultaneous circulation of pandemic H1N1 and influenza B viruses. Moreover, data from both systems enables to calculate age- and sex-adjusted rates, but by SSD deeper analysis can be done, including identification SARI or fatal cases risk groups.
From the above data it can be said that SSD completely reflects trends received from RSD, but gives more precise explainable picture of situation. Moreover, in case of influenza timely detection of new virus or any unusual increasing of cases providing by sentinel surveillance is more important, than retrospective incidence rates. Revealing of conditions led to developing of SARI or fatal cases is useful to determine risk-groups to be vaccinated against influenza.

Key words: influenza, incidence, sentinel, surveillance system

P94

The human Mx locus exhibits potent antiviral activity in an inducible transgenic mouse model

C. Deeg[1*], C. Kallfass[1], C. Nürnberger[1,2], P. Stäheli[1]

[1] University of Freiburg, Virology, Freiburg, Germany; [2]Spemann Graduate School of Biology and Medicine, University of Freiburg

The interferon-regulated Mx proteins are potent antiviral effector molecules. The murine Mx1 protein mediates protection against influenza A and B viruses as well as against other orthomyxoviruses such as Thogoto virus. In cell culture, the human MxA protein is active against an even broader range of RNA viruses, whereas the human MxB protein seems devoid of antiviral activity. The murine and the human Mx proteins exhibit ~80% sequence identity on protein level but differ in intracellular localisation and might therefore act differently. To investigate the function of the human Mx proteins *in vivo*, we created a novel transgenic mouse that carries the entire human Mx locus including the complete MxA and MxB coding sequences as well as adjacent regulatory elements of both genes.
We found that embryonic fibroblasts from such animals as well as live transgenic mice synthesize high levels of MxA upon IFN-α treatment, whereas basal expression levels were minute. Viral challenge experiments demonstrated that the Mx-transgenic mice are protected from lethal infections with influenza A virus and Thogoto virus. Consistently, viral titers in the lung were strongly reduced in transgenic mice compared to non-transgenic littermates.
In conclusion, our novel transgenic mouse represents a suitable model for investigating the *in vivo* antiviral activity of the human Mx system.

Key words: Innate immunity, human MxA, transgenic mouse model, interferon-inducible, Influenza A virus

P95

The Combined Action of Influenza Virus and *Staphylococcus aureus* Panton-Valentine Leukocidin Provokes Severe Lung Epithelium Damage

S. Niemann[1,3*], C. Ehrhardt[2], E. Medina[4], K. Warnking[2], L. Tuchscherr[1,3], V. Heitmann[1], S. Ludwig[2], G. Peters[1,3], B. Löffler[1,3]

[1]Institute of Medical Microbiology; [2]Institute of Molecular Virology and [3]Interdisciplinary Center of Clinical Research, University Hospital of Münster; [4]Helmholtz Center for Infection Research, Braunschweig, Germany

S. aureus necrotizing pneumonia is a life-threatening disease that is frequently preceded by an influenza infection. However the precise interplay between both pathogens, *S. aureus* and influenza virus, remains to be elucidated.
In this study, we present a model that explains how influenza virus and the pore-forming *S. aureus* toxin Panton-Valentine-leukocidin (PVL) act together to cause necrotizing pneumonia. We found that PVL induced rapid cell death in neutrophils, which was augmented by coinfection with influenza virus. Epithelial cells strongly up-regulated chemokine expression in response to influenza infection. Incubation of epithelial cells with supernatants from PVL-treated neutrophils resulted in a dose-dependent cell detachment and disruption of the epithelial monolayer. Furthermore, intranasal instillation of mice with supernatants from PVL-damaged human neutrophils caused extensive airway epithelial exfoliation and tissue damage with signs of necrotizing pneumonia. The devastating effect on lung epithelium was completely prevented by adding a protease inhibitor cocktail or human serum, indicating that destruction is caused by uncontrolled release of massive amount of neutrophil proteases in the respiratory space. Additionally, human serum conferred protection against PVL-induced cytotoxicity in neutrophils, which was mediated by PVL-neutralizing antibodies. These findings can explain that necrotizing infections mainly develop in serum-free spaces such as pulmonary alveoli.
Taken together, we propose a model where influenza virus and superinfection with PVL-producing *S. aureus* lead to fatal necrotizing pneumonia. Modulation of influenza induced chemotaxis, PVL-induced neutrophil cytotoxicity as well as the uncontrolled release of proteases may represent possible targets for therapeutic interventions.

Key words: S. aureus necrotizing pneumonia; pore-forming toxin PVL; superinfection with influenza virus; epithelial activation; uncontrolled neutrophil cell death

P96

Polymerase mutations promoting adaptation of avian influenza virus of subtype H9N2 to mammals.

Hanna Sediri[1*], Hans-Dieter Klenk[1]

[1]Institut for virology, Hans-Meerwein Strasse 2, 35043 Marburg, Germany

Aquatic birds are the natural reservoir of influenza A viruses. Interspecies transmission can occur from aquatic birds to terrestrial birds or to mammals where, upon adaptation, some of these viruses may cause devastating outbreaks in domestic poultry or cause human pandemics. Avian influenza A viruses of subtype H9N2 are frequently observed in poultry in Asia and the Middle East. They have occasionally been passed from poultry to humans and have therefore pandemic potential.
There is evidence that PB2 is an important determinant of host range and that mutations E627K, D701N, S714I or S714R may promote adaptation to mammals.

In the present study we have introduced these mutations in the PB2 of avian H9N2 isolates, A/Quail/Shantou/2061/2000 and A/Quail/Shantou/782/2000. Employing minireplicon assays we observed a 15-fold increase in polymerase activity with mutation E627K, and a 5-fold increase with mutations D701N, S714I and S714R, respectively. We also compared the impact of these mutations with H7N7 and pandemic H1N1 virus, and observed that the increase in polymerase activity was most distinct in H9N2 viruses.

Thus we have obtained evidence that mutations in the polymerase subunits PB2 may promote adaptation of avian H9N2 viruses to mammalian hosts.

Key words: polymerase, adaptation, H9N2 viruses

P97

Newly designed furin-specific inhibitors exhibit potent inhibitory efficacy against highly pathogenic influenza viruses

Yinghui Lu[1*], Gero Becker[2], Torsten Steinmetzer[2], Wolfgang Garten[1]

[1]Institut für Virologie, Philipps-Universität Marburg; [2]Institut für Pharmazeutische Chemie, Philipps-Universität Marburg

Furin is a ubiquitously expressed mammalian subtilisin-like protease. It is responsible for the activation of highly pathogenic avian influenza virus strains of the subtypes H5 and H7. It cleaves the hemagglutinin precursor HA0 into its subunits HA1 and HA2 at the C-terminus of the multibasic amino acid sequence motif -R-X-R/K-R-. Peptidomimetics containing the furin recognition motif were designed and synthesized as specific furin inhibitors and considered as attractive antiviral drug candidates against highly pathogenic influenza viruses. We examined virus propagation in presence of various furin inhibitors added to cell cultures infected with highly pathogenic influenza viruses, i. e., a mouse adapted influenza virus SC35M (H7N7) or A/fowl plague virus/Rostock/34 (H7N1). To optimize the antiviral effects we treated infected cells with the most efficacious furin inhibitor in a combination with the neuraminidase inhibitor oseltamivir. The inhibition effect of the combination with both inhibitors proved to be synergistic. Our data suggest that a combined therapy with a furin-inhibitory peptidomimetic and a neuraminidase inhibitor may be a very effective and viable strategy for the treatment of humans afflicted with a high pathogenic avian influenza virus infection.

Key words: furin, inhibitor

P98

Host-specific differences in membrane fusion activity of influenza A viruses

J. Baumann[1*], N. Monougu[1], H. D. Klenk[1], M. Matrosovich[1]

[1] Institute of Virology, Philipps University, Marburg, Germany

The repeated occurrence of zoonotic influenza A viruses in the human population highlights the necessity to understand which viral properties contribute to interspecies transmission. Viral HA-mediated recognition of sialic acid receptors in humans is well known to be essential for the transmission. Less is known about the potential role of the HA membrane fusion activity. To address this question, we compared fusogenic properties of influenza viruses isolated from birds, pigs and humans.

Using modified high-sensitivity hemolysis assay, we found that the pH optimum of membrane fusion of swine influenza viruses typically is 0.2 to 0.5 pH units higher when compared to avian and human viruses. In agreement with the hemolysis data, avian and human viruses were more efficiently neutralized than swine viruses by the lysosomotropic agent ammonium chloride, which interferes with the acidification of low pH cellular compartments (avian, human: $IC_{50\%}$: 0.6-1.0 mM NH_4Cl; swine: 1.5- 2.5 mM NH_4Cl). These results suggest that pH optimum of HA-mediated membrane fusion varies depending on the host species and can be a factor restricting avian-to-swine and swine-to-human transmission of influenza viruses.

To identify amino acids responsible for alteration of the fusion activity of the HA during avian-to-swine adaptation, HA sequences of the Eurasian avian-like swine viruses were compared with HA sequences of their putative avian precursors. Eight amino acids showed distinct host-specific distribution between the avian and swine HAs. Based on their location, four amino acids could potentially affect membrane fusion activity. Phenotypic characterization of individual amino acid substitutions are in progress.

Key words: pH-Optimum, Hemagglutinin, Hemolysis, pig, bird

P99

Antiviral activity of chicken interferon-λ in chickens

A. Reuter[1,4], D. Rubbenstroth[1], S.M. Soubies[1*], S. Härtle[3], P. Stäheli[1]

[1]Department of Virology, University of Freiburg, Freiburg, Germany; [2]Institute for Virology, University of Veterinary Medicine Hannover, Hannover, Germany; [3]Department of Veterinary Sciences, University of Munich, Munich, Germany; [4]International Max Planck Research School for Molecular and Cellular Biology, Freiburg

In mammals, type III interferons have been shown to play a crucial role in epithelial defense against viral infections. In contrast, little is known about the role of interferon λ (IFN-λ) in the antiviral innate response in chickens (*Gallus gallus*).
To better understand responsiveness to this cytokine in various tissues, we first analyzed its antiviral activity in chicken cell lines as well as in primary cells from epithelia-rich organs. Contrary to the fibroblast-derived cell line DF-1, the lung-derived cell line CLEC-213 as well as primary kidney cells showed protection from Vesicular stomatitis virus (VSV) infection when pre-treated with recombinant IFN-λ.
We then analyzed the degree of antiviral protection elicited by retrovirally-expressed IFN-λ *in ovo*. Transgenic embryos constitutively expressing IFN-λ showed a dramatic protection to infection with various viruses, including highly pathogenic avian influenza virus (HPAIV) H5N1 infection. Protection correlated with upregulated transcript levels of interferon-stimulated gene Mx in various tissues as well as in chorio-allantoic membrane, suggesting an activation of the antiviral innate response. Consistently with the *in ovo* data, administration of recombinant IFN-λ *in vivo* resulted in a clear Mx upregulation in epithelia-rich tissues, such as kidney, trachea and lung.
In summary, these results suggest a prominent antiviral activity of IFN-λ in chickens. Similar to what is known in mammals, this activity appears to be restricted to epithelia-rich tissues.

Key words: chicken, innate immunity, interferon-lambda, antiviral activity

P100

Influenza A NS1 protein is required for efficient segment 7 mRNA nuclear export

C. Pereira[1*], E. Read[1], H. Wise[2], M. Amorim[3], P. Digard[1,2]

[1] Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK; [2] The Roslin Institute, Edinburgh University, Easter Bush, Midlothian EH25 9RG, UK; [3] Instituto Gulbenkian de Ciencia, Rua da quinta grande 6, P-2780-156 Oeiras, Portugal

The RNA genome of influenza A virus is transcribed in the cell nucleus, necessitating nuclear export of viral mRNA. The cellular TAP/NXF1-dependent mRNA export pathway has been shown to be involved in the export of certain viral mRNAs but how they are recruited to this pathway is unknown.
Using a minireplicon transfection system to recreate viral RNPs with single vRNA templates, the requirements for segment 7 mRNA export were studied. Fluorescence *in situ* hybridization (FISH) showed that under these circumstances, segment 7 mRNA was largely retained in the nucleus, suggesting an additional viral protein was needed for its export. Addition of further viral proteins identified this as NS1 and analysis of NS1 mutants showed that intact RNA-binding and effector domains were required for export activity. Protein analyses showed a reduction in both M1 and M2 protein expression in cells infected with NS1 mutant viruses. Furthermore GFP trap pull downs showed that while TAP/NXF1 bound WT NS1 and segment 7 mRNA, NS1 mutants unable to promote export of segment 7 mRNA were also deficient in these interactions.
We conclude that NS1 is required for efficient export of M1 mRNA, potentially by acting as an adaptor protein between the viral RNA synthesis machinery and cellular export pathway.

Key words: mRNA nuclear export, NXF1

P101

Identification of interferon-β producing cells in influenza A virus-infected mouse lungs

C.Kallfass[1*], S. Lienenklaus[2], P.Staeheli[1]

[1]Department of Virology, University of Freiburg, 79104 Freiburg, Germany; [2]Molecular Immunology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany;

Interferon (IFN)-β contributes to innate immunity in mammalian hosts. Information about the *in vivo* source of IFN-β during viral infections remains incomplete. To identify the cell types responsible for IFN-β production, we employed a reporter mouse which expresses firefly luciferase under control of the IFN-β promoter, and stained organ sections of virus-infected mice with antibodies for firefly luciferase. Here, we studied the IFN-β response in lungs of mice infected with the H7N7 influenza A virus strain SC35M and its attenuated derivative SC35M-ΔNS1 which lacks the IFN-antagonist factor NS1. Luciferase-positive cells were detected in the lung of reporter mice infected with either SC35M or SC35M-ΔNS1, whereas no such cells were observed in lungs of uninfected mice. Double staining experiments in which staining for luciferase was combined with staining for cell-type specific markers or viral antigen indicated that lung epithelial cells are the main source of IFN-β during infection with SC35M-ΔNS1, whereas a variety of different cell types including macrophages, dendritic cells and epithelial cells contributed to IFN-β synthesis during infection with wild-type SC35M. We confirmed these findings using tissue-specific reporter mice that express the virus-inducible luciferase reporter gene solely in defined cell types. Our results demonstrate that infected lung epithelial cells are a rich source of IFN-β. However, they also show that influenza virus employs the NS1 protein to efficiently restrict the antiviral potential of these cells.

Key words: IFN-β production; IFN-β reporter mouse

P102

Influenza-virus infection impacts on alveolar edema clearance: role of virus- and host factors in regulation of alveolar epithelial cell ENaC and Na,K-ATPase expression

C.Becker[1*], S.Budinger[2], W.Seeger[1], J.Lohmeyer[1], R.Morty[3], S.Herold[1]

[1]University of Giessen Lung Center, Giessen; [2]Northwestern University, Chicago; [3]Max-Planck-Institute for Heart and Lung Research, Bad Nauheim

Influenza A viruses (IV) can cause primary viral pneumonia in humans resulting in acute lung injury (ALI/ARDS) with fatal outcome. IV-induced ALI is accompanied by the accumulation of edema fluid in the alveolar compartment, which under normal conditions is cleared along a sodium gradient established by basolateral Na,K-ATPase and the apical epithelial sodium channel (ENaC). IV-infection was found to be associated with a decrease in alveolar fluid clearance (AFC). Therefore we aim to investigate the regulation of ENaC and Na,K-ATPase in IV-infection by viral and host factors to characterize the molecular mechanisms affecting AFC .

Real-time PCR revealed the presence of Na,K-ATPase subunit isoforms α1, α2, α3, β1 and γ in murine alveolar epithelial cells (AEC), none of which was regulated on mRNA level upon IV-infection. ENaC subunits were regulated on mRNA level 12 hours post infection (hpi) and 24hpi in a dose-dependent manner. Na,K-ATPase protein levels were significantly decreased in the time-course of IV-infection in AEC monoculture. Cocultivation of AEC with infected alveolar macrophages further enhanced the degradation of Na,K-ATPase protein levels. Both surface fraction analysis based on Biotin-Streptavidin-pulldown of surface proteins, and immunofluorescence revealed distinct changes in cellular localization patterns with a decrease of basolaterally located Na,K-ATPase in the time-course of infection.

We provide evidence that viral and host factors directly impair edema clearance in IV-induced ALI by affecting ENaC and Na,K-ATPase expression levels and localisation. Defining the molecular pathways underlying these effects might provide potential targets for new treatment strategies to increase edema clearance in IV-induced ALI.

Keywords: Na,K-ATPase, ENaC, edema clearance, Influenza A virus

P103

Alteration of infectivity and antigenicity by host adaptation of the first Japanese A/H1N1pdm09 isolate

Hideki Asanuma[1,2*], Akira Ainai[1,2], Masatsugu Obuchi[1], Takato Odagiri[1], Masayuki Shirakura[1], Eri Nobusawa[1], Tetsutaro Sata[1], Hideki Hasegawa[1,2], Masato Tashiro[1]

[1]Center for Influenza Virus Research, National Institute of Infectious Diseases
[2]Dept. of Pathology, National Institute of Infectious Diseases

A/Narita/1/2009 (A/N) is the first A/H1N1pdm virus isolated in Japan. To understand the effects by host-specific adaptation, A/N was passaged in MDCK cells, embryonated hen eggs and BALB/c mice, respectively. A/N which had been serially passaged in cells, eggs or mice acquired the ability to grow efficiently in each respective host. Moreover, A/N after passages in mice became high pathogenic in mice. All of the passaged A/N had amino acid changes between residues 153 and 157 in HA1 while additional substitutions occurred depending on different hosts; Q223R only in egg-passaged and D222G only in mice-passaged viruses, respectively. Although these changes in virus growth and pathogenicity are likely related to the amino acid substitutions in the hemagglutinin, possible effects of amino acid changes in other genes are under investigating. In addition, post-infection ferret sera against either egg-isolated or cell-isolated A/N showed reduced HI titers to the all of the passaged viruses, while such a reduction was not found when using mouse antisera. In conclusion, the A/N virus could differentially adapt to new hosts upon extended passages, as indicated by an increase in virus replication and antigenic change, that was associated with host-dependent amino acid substitutions.

P104

Comparative analysis of mammalian ganglioside receptors of influenza A viruses and soluble hemagglutinins

A. Hucke[1], D. Eberhardt[1], G. Pohlentz[1], A.-K. Sauer[2], G. Herrler[2], C. Ehrhardt[3], S. Ludwig[3], W. Storck[1], I. Meisen[1], M. Mormann[4], H. Karch[1], J. Müthing[1*]

[1]Institute for Hygiene, University of Münster, Münster, Germany; [2]Institute of Virology, University of Veterinary Medicine, Hannover, Germany; [3]Institute of Molecular Virology, ZMBE, Münster, Germany; [4]Institute of Medical Physics and Biophysics, University of Münster, Münster, Germany

Viral hemagglutinins (HAs) bind to sialylated glycoconjugates such as gangliosides (sialylated glycolipids) and/or sialoglycoproteins that serve as receptors for influenza A viruses in the plasma membrane of target cells. Although sialic acid-mediated cellular interaction of HA represents the initial step in the infection process, the explicit receptors of HA are largely unknown. To gain knowledge on virus binding specificity, we explored the interaction of mammalian gangliosides with recombinant soluble H7 and H9 hemagglutinins of avian origin and porcine H1N1 influenza A virus strains. Gangliosides were chosen because their assembly in lipid rafts – dynamic membrane microdomains enriched in cholesterol and glycolipids – is believed to be a key requirement for binding and subsequent internalization of virions. Specific anti-ganglioside antibodies, which are able to discriminate between α2-3- and α2-6-sialylated gangliosides, were used as controls in comparative overlay binding assays. Different binding pattern of swine H1N1 influenza virus was observed in comparison to human H3N2 influenza A viruses towards gangliosides haboring α2-3- or α2-6-linked sialic acid as determined by electrospray ionization mass spectrometry (ESI MS). Recombinantly produced soluble H7 and H9 hemagglutinins exhibited a distinct specificity towards human and animal gangliosides, giving evidence for preferential binding of H7 to α2-3-sialylated gangliosides with Galβ1-4GlcNAc-core structures. Thus, beyond the type of terminally linked sialic acid (α2-3 *versus* α2-6) of glycan ligands we have to consider also the carbohydrate backbone structure, which may contribute to favorable HA-receptor interaction. Future experiments have to confirm the functional importance of ganglioside receptors in lung cells.

Key words: glycoconjugates, sialic acid, protein-carbohydrate interaction

P105

Using quantitative proteomic analysis of recombinant influenza A virus to define the interactome of the viral NS1 protein in human lung cells

K. Paki[1], T. Schwecke[2], A. Sadewasser[1], T. Wolff[1*]

[1] Div. of Influenza/Respiratory Viruses; Robert Koch-Institut, Nordufer 20, 13353 Berlin;
[2]Centre for Biological Security 6; Robert Koch-Institut, Nordufer 20, 13353 Berlin

The influenza A virus NS1 protein modulates various cellular processes including the antiviral interferon response, cellular RNA metabolism and phosphoinositide 3-kinase signalling. NS1 has a modular structure consisting of an N-terminal RNA binding and dimerization domain (aa 1-73) and a C-terminal effector domain (aa 74-231). We wish to characterize the pleiotropic modes of action for the viral NS1 protein by defining the whole ensemble of cellular binding partners in the infected host cell using quantitative proteomic methods.
Towards this goal we employed a recombinant influenza A virus expressing a fusion protein including NS1 amino acids 1-104 and the autofluorescent GFP protein combined with stable isotope labeling by amino acids in cell culture (SILAC). Lysates of infected cells were subjected to immunoprecipitation through the GFP trap method followed by detection of bound proteins using high resolution mass spectrometry by coupled LC-MS/MS. This resulted in the identification of a distinct set of high confidence cellular factors. The list contained proteins such as PABP1 and hnRNP-F that have previously been described to bind to the NS1 protein. In addition, several novel NS1 interactor candidates were found. We will present a comprehensive analysis of novel NS1 binding proteins and will discuss the potency of novel proteomic tools to define host virus interactions.

Key words: influenza A virus, NS1, GFP trap, interactome, SILAC

P106

Sialyl Lewis^x gangliosides: novel receptors of human H3N2 influenza A viruses

I. Meisen[1,2*], T. Dzudzek[1,3], C. Ehrhardt[2,3], S. Ludwig[2,3], M. Mormann[4], H. Karch[1], J. Müthing[1,2]

[1]Institute for Hygiene, University of Münster, Münster, Germany; [2]Interdisciplinary Center for Clinical Research (IZKF), University of Münster, Münster, Germany; [3]Institute of Molecular Virology, ZMBE, University of Münster, Münster, Germany; [4]Institute of Medical Physics and Biophysics, University of Münster, Münster, Germany

An emerging number of viruses has been described to exploit sialic acid-carrying glycosphingolipids (gangliosides) as targets on mammalian host cells initiating infections through protein- carbohydrate (pathogen-host) interactions. Gangliosides from human granulocytes, which are the second most abundant cells in peripheral blood and involved in phagocytosis of pathogens, were probed for their binding potential to human H3N2 virus strains A/Victoria/3/75 and A/Hiroshima/52/2005. The preponderant gangliosides with nLc4Cer and nLc6Cer cores carrying α2-3- or α2-6-linked *N*-acetylneuraminic acid (Neu5Ac), gave largely negative results in virus thin-layer chromatography (TLC) overlay assays. On the other hand, highly polar gangliosides with low TLC mobility exhibited strong interaction with both H3N2 viruses indicating preferential binding of the pathogens to fucosylated monosialogangliosides with extended neolacto (nLc)-series core oligosaccharides. Full structures of the virus receptors were obtained using monoclonal anti-sialyl Lewis^x (sLe^x) antibodies with binding specificity to the Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc-R epitope, which were employed in comparative binding studies, together with electrospray ionization mass spectrometry analysis. Preferentially recognized sLe^x-gangliosides were those with nLc8, nLc10 and nLc12 backbone, harboring sphingosine (d18:1) and a fatty acid with variable chain length in the ceramide lipid anchor and one or two additional internal fucose molecules in the oligosaccharide chain. These specific binding requirements beyond terminally α2-3-linked sialic acid with regard to carbohydrate chain length and subterminal fucosylation of glycans with Galβ1-4GlcNAc-residues open new questions of the functional role of sLe^x-gangliosides as receptors for the H3N2 subtype of influenza A viruses.

Key words: electrospray ionization mass spectrometry, polyglycosylceramides, sialyl Lewis^x, TLC overlay assays

P107

Pigs as an intermediate host for the adaptation of Asian lineage H5N1 highly pathogenic avian influenza (HPAI) viruses to mammals

Brandon Z. Londt*, Sharon M. Brookes, Bethany J. Nash, Alejandro Núñez, Michael D. Kelly and Ian H. Brown

Virology Department, Animal Health and Veterinary Laboratories Agency (AHVLA), Addlestone, Surrey, KT15 3NB, United Kingdom

Pigs could play a role in the adaptation of Asian lineage highly pathogenic avian influenza (HPAI) virus to mammals, allowing this highly virulent variant to become established within livestock and/or the human population. To determine the feasibility of this, A/turkey/Turkey/1/05 (H5N1) HPAI virus was passaged *in vitro* in pig cells alone, in the presence of antiserum to the homologous virus or porcine IFN-γ. Mutations were identified for viruses passaged in pig cells in the presence of immune pressure only. The infectivity of the mutated viruses was assessed using *ex vivo* pig bronchi and lung organ cultures. Although higher levels of virus were detected in organ cultures infected with viruses passaged in pig cell lines when compared with the wild-type parental viruses, similar results were observed in nasal shedding, tissue tropisms and viral loads when pigs were infected experimentally with these viruses. When naïve pigs were placed in contact with chickens and Pekin ducks infected with the wild type virus, transmission to the pigs was unsuccessful. These results suggest that although mammalian adaptive mutations may be acquired by H5N1 HPAI viruses, this may not translate necessarily into an increase in infectivity or disease in pigs *in vivo*. Notwithstanding the role direct infection in humans may play, the potential for pigs to generate mammalian specific mutations in H5N1 HPAI viruses is low since transmission of these viruses to pigs from infected birds is limited. The potential role of pigs as an intermediate host for these HPAI H5N1 viruses may be less significant than previously expected.

Key words: H5N1, HPAI, pigs, Asian lineage

P108

Macrophage-expressed IFN-β mediates apoptotic alveolar epithelial injury in severe influenza virus pneumonia

Katrin Högner*, Thorsten Wolff, Stephan Pleschka, Achim D. Gruber, Ulrich Kalinke, Dieter Walmrath, Johannes Bodner, Stefan Gattenlöhner, Peter Lewe-Schlosser, Mikhail Matrosovich, Werner Seeger, Juergen Lohmeyer and Susanne Herold

Influenza viruses (IV) cause pneumonia in humans with progression to lung failure and fatal outcome. Dysregulated release of cytokines including type I interferons (IFNs) has been attributed a crucial role in immune-mediated pulmonary injury during severe IV infection. Using ex vivo and in vivo IV infection models we demonstrate that alveolar macrophage (AM)-expressed IFN-β significantly contributes to IV-induced alveolar epithelial cell (AEC) injury by autocrine induction of the pro-apoptotic TNF-related apoptosis-inducing ligand (TRAIL). Importantly, TRAIL was highly upregulated in AM of patients with pandemic H1N1 IV-induced lung failure. Elucidating the cell-specific underlying signalling pathways revealed that IV infection induced IFN-β release in AM in a protein kinase R- (PKR-) and NF-κB-dependent way. Autocrine signalling via the macrophage type I IFN receptor (IFNAR) resulted in increased expression and release of TRAIL which induced apoptosis of IV-infected and non-infected AEC in ex vivo co-cultures, and AEC apoptosis induction was shown to be strictly dependent on the macrophage PKR/IFNAR/TRAIL axis. Bone marrow chimeric mice lacking these signalling mediators in resident and lung-recruited AM and mice subjected to alveolar neutralization of IFN-β and TRAIL displayed reduced alveolar epithelial cell apoptosis and attenuated lung injury during severe IV pneumonia. Together, we demonstrate that macrophage-released type I IFNs, apart from their well-known anti-viral properties, contribute to IV-induced AEC damage and lung injury by autocrine induction of the pro-apoptotic factor TRAIL. Therapeutic targeting of the macrophage IFN-β-TRAIL axis might therefore represent a promising strategy to attenuate IV-induced acute lung injury.

P109

Anti-influenza virus activity of nucleoside inhibitors of the influenza virus polymerase

E. Vanderlinden[1*], S. Meneghesso[2], J. Balzarini[1], C. McGuigan[2], L. Naesens[1]

[1]Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium; [2]Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK

Several base- and/or sugar-modified UTP and GTP derivatives were observed to exert an inhibitory effect on the influenza polymerase, using an RNA elongation assay with virion-derived vRNPs. The most potent inhibitors were 5-bromo-UTP (IC_{50}: 7.1 µM), 2'-fluoro-5-methyl-UTP (IC_{50}: 9.5 µM), 2'-fluoro-2'-deoxy-UTP (IC_{50}: 14 µM), 7-deaza-GTP (IC_{50}: 4.1 µM) and 2'-fluoro-2'-deoxy-GTP (IC_{50}: 3.7 µM). These data provide a rationale to design nucleoside analogues for selective inhibition of influenza virus in cell culture. To improve the intracellular bioavailability and activation, the phosphoramidate ProTide technology, delivering the activated (monophosphorylated) nucleoside analogue, was applied to the modified uridines and guanosines. We identified two 2'-fluoro-2'-deoxyuridine ProTides with moderate antiviral activity in influenza virus-infected cells, while the parent nucleoside analogue was inactive. To the 2'-fluoro-2'-deoxy-guanosine, we applied the double prodrug approach, combining a ProTide motif and a 6-O-substituted guanine. The lipophilicity of the latter substituent further enhances the cellular uptake of the ProTide. In a PCR-based virus yield assay, the ProTides derived from 6-O-methoxy-2'-fluoro-2'-deoxy-GMP, 6-O-ethoxy-2'-fluoro-2'-deoxy-GMP and 6-Cl-2'-fluoro-2'-deoxy-GMP displayed EC_{99} values of 15, 14 and 12 µM, respectively, while the corresponding nucleoside analogues showed no activity at 100 µM.

The use of 5-methyl-UTP, 5-bromo-UTP and 7-deaza-GTP as alternative substrates for the natural UTP or GTP substrate, was demonstrated in the influenza polymerase assay. The RNA elongation reaction was shown to proceed equally efficient when UTP was replaced by 5-methyl-UTP or 5-bromo-UTP. A similar observation was made with 7-deaza-GTP. The implications of their incorporation on viral RNA transcription and translation are the subject of current investigation.

Key words: influenza, polymerase, antiviral, ProTide, nucleoside

P110

Mutations in the cytoplasmic tail of HA in Influenza virus – with focus on S-Acylation

Katharina Brett*, Larisa Kordyukova[1], Lars Möller[2], Michael Laue[2], Michael Veit[3]

[3]Institut für Immunologie und Molekularbiologie, Fachbereich Veterinärmedizin, Freie Universität Berlin; [1]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia; [2] Centre for Biological Safety 4, Robert Koch Institute, Berlin, Germany

Hemagglutinin (HA) of Influenza A virus is post-translationally modified by S-acylation at typically three cysteines, one in the transmembrane region and two in the cytoplasmic tail. This modification is essential for virus viability. Mass spectrometry revealed that the cysteines in the cytoplasmic tail are palmitoylated, while cysteines in the transmembrane region carry stearate.
We hypothesized that the residues surrounding the acylation sites contain consensus signals for S-acylation or determine the fatty acid specificity. To investigate this, we mutated conserved residues in HA's cytoplasmic tail to generate recombinant viruses by reverse genetics in the WSN background. Mutation of the conserved glycine-557 (situated three residues downstream of the transmembrane cysteine) to alanine affected virus growth only marginally and mass spectrometry revealed no change in acylation, neither its stoichiometry nor fatty acid selectivity was altered. Mutation of glutamine-560, directly preceding the second acylated cysteine, to glutamic acid resulted in a severely growth-compromised virus. Electron microscopy did not show aberrant morphology of either virus. Mutants G557Stop and I563Q could not be rescued.
In combination, the results show that even single-residue changes in the cytoplasmic tail of HA strongly hamper virus growth. Further site-directed mutations in this region will clarify whether the amino acid sequence in the vicinity of the modified cysteines or the location of the cysteine relative to the transmembrane region is responsible for differential attachment of fatty acids.

Key words: Hemagglutinin, S-Acylation, reverse genetics, cytoplasmic tail

P111

Antigenic variation of H2N2 influenza viruses during 11 years of evolution

Martin Linster[1*], Björn Koel[1], Theo Bestebroer[1], David F. Burke[2],Albert D.M.E.Osterhaus[1] Derek J. Smith[2], Sander Herfst[1], RonA.M. Fouchier[1]

[1]Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands
[2]Department of Zoology, University of Cambridge, Cambridge, United Kingdom

Influenza A viruses of the H2 subtype originate like all other subtypes from the aquatic bird reservoir. In 1957,an H2N2 influenza A virus was first introduced into the human population sparking the 'Asian' pandemic. This virus replaced the previously circulating H1N1 viruses and was introduced into a human population naïve to this newly emerged subtype. In this study we address the question how H2N2 viruses drifted antigenicallyto ensure sustained infection of human individuals and to escape antibody pressure.
A set of H2N2 viruses spanning the total time of circulation in the human population (1957-1968) was used in this study. Antisera against selected antigens were raised in ferrets and tested in hemagglutinin inhibition (HI) assays against wildtype and mutant H2N2 viruses. HI results were analyzed using multiple dimensional scaling methods to generate antigenic maps.
Antigenic drift of H2N2 influenza A viruses can be described as amore or less linear evolutionary trajectory. Single amino acid substitutions near the receptor binding site of the HA molecule change the recognition of the virus by antibodies dramatically. A limited number of mutations can describe the 11 years of antigenic evolution.
Other mutations that occurred and were fixed in H2N2 viruses must be considered to have different functions. Vaccine development and surveillance of avian influenza viruses can greatly profit from theses findings.

Key words: antigenic drift, H2N2, receptor binding site, antigenic mapping

P112

P38 MAPK signaling pathway is involved in regulating interferon-λ1-mediated immune responses after RNA stimulation

Miao Jiang[1,2,3*], Pamela Österlund[1], Minna M. Poranen[2,3], Dennis H. Bamford[2,3], Ilkka Julkunen[1]

[1]Department of Vaccination and Immune Protection, National Institute for Health and Welfare, FIN-00271 Helsinki, Finland; [2]Institute of Biotechnology,
University of Helsinki, FIN-00014 Helsinki, Finland; [3]Department of Biosciences, University of Helsinki, FIN-00014 Helsinki, Finland

Virus infection can induce multiple innate immune responses, including interferon (IFN)-mediated antiviral responses. Viral nucleic acids are recognized by different pattern recognition receptors and this induces type I (IFN-α/β) and type III IFN (IFN-λ1-3) gene expression through multiple signaling pathways. Although much has been studied regarding to the structure and the function of IFN genes, there are still many details unknown or unverified concerning signaling pathways involved in the activation and regulation of IFN gene expression, especially type III IFN genes. Here, we demonstrate that P38 mitogen-activated protein kinase (MAPK) signaling pathway is involved in regulating IFN-λ1 gene expression after different RNA stimulations in human monocyte-derived dendritic cells (moDCs), as adding P38 inhibitor can totally abolish the mRNA expression of IFN-λ1. Moreover, ISRE and NF-κB transcription factor binding sites (TFBSs) are essential for activation of IFN-λ1 promoter by RNAs, as could be confirmed from our IFN-λ1 promoter mutant experiments. P38 inhibitor can also hinder the phosphorylation of IRF3 and the binding of IRF3 and p50/p65 transcription factors to ISRE and NF-κB TFBSs after viral RNA stimulation in moDCs. Furthermore, overexpression studies in HEK cells showed that p38 regulates IFN-λ1 promoter activation through RIG-I-MAVS-IRF3 or TRIF-IRF3 signaling pathway, but not through MyD88-IRF7 signaling pathway. Altogether, our data establish that p38 Map kinase pathway has crosslink with RIG-I and TRIF signaling pathway and is involved in regulating the activation of IFN-λ1 gene promoter through the binding of transcription factors to ISRE and NF-κB TFBSs, to further mediate antiviral responses.

Key words: interferon, p38, transcription factor binding site, RNA stimulation, signaling pathway

P113

Hemadsorption activity of the neuraminidase of H1N1/09 pandemic influenza virus and its swine precursors

V. Czudai-Matwich[1*], J. Uhlendorff[1], V.Laukemper[1], T.Gerlach[1], H-D. Klenk[1], M. Matrosovich[1]

[1]Philipps-University Marburg, Institute of Virology, Marburg, Germany

The neuraminidases (NA) of avian influenza A viruses contain, in addition to the catalytic site, a second sialic acid-binding site (hemadsorption site, HAD) which serves to enhance catalytic activity of the NA. By contrast, the NAs of the 1918 and 1957 pandemic viruses contained mutations in the HAD site and displayed reduced HAD activity suggesting these alterationsare needed forthe emergence of pandemic viruses from their avian precursors. The NA of the 2009 pandemic virus (H1N1pdm) originated from the Eurasian avian-like swine (EASw) virus. This prompted us to study evolution of the HAD activity of the N1 NA in pigs and humans by comparing phylogenetically related NAs of H1N1 avian viruses, their descendant EASw viruses, and H1N1pdm.

We found that the NA of H1N1pdm lacks HAD activity. The NAs of EASw viruses lost this activity at some time point between 1981 (A/swine/Germany/2/81, HAD-positive) and 1992 (A/swine/England/195852/92, HAD-negative), i.e., several years after transmission of the avian precursor to pigs and long before the transmission of the swine precursor to humans. By comparing the NA sequences, we identified four mutations that distinguished HAD-positive avian and early EAsw virus NAs from those of their EAswdescendants and tested effects of individual mutations by site-directed mutagenesis. Our data suggest that the substitution Y344N, which is shared by the N1 NAs of late EASw viruses, classical swine and human viruses is a major determinant of their HAD-negative phenotype. We assume that this mutation can represent a marker of mammalian adaptation of N1 NAs.

Key words: Influenza, H1N1pdm, Neuraminidase, Hemadsorption

P114

Emergence of novel influenza A(H3N2) variants in Germany during 2011-2012

M. Wedde[1*], S. Hafemann[1], S. Tietze[1], B. Schweiger[1]

[1]Robert Koch-Institut, National Reference Centre for Influenza, Berlin, Germany

Influenza A(H3N2) viruses predominated during 2011-2012. Antigenic and genetic characterization was performed by haemagglutination inhibition (HI) and cycle sequencing, respectively. The antigenic profile of a minority of A(H3N2) viruses could not be determined. These viruses were further characterized by the rapid and high throughput pyrosequencing (PSQ) technique.

Phylogenetic analysis of the haemagglutinin (HA) genes revealed that the A(H3N2) viruses belonged to four genetic groups. Group 3 viruses predominated (85%) and were characterized by the co-circulation of three different subgroups. A/Victoria/361/2011-like (3C), A/England/259/2011-like (3B) and A/Stockholm/18/2011-like (3A) viruses showed a prevalence of 51%, 24%, and 10%, respectively. Subgroup 3B and 3C viruses were detected for the first time in Germany. The antigenic profile of all co-circulating variants was comparable. The majority of those viruses were antigenically similar to the vaccine strain A/Perth/16/2009. PSQ analysis of the HA genes of viruses with unknown antigenic profile revealed that the majority of viruses belonged to the A/England/259/2011 subgroup (50%) which is characterized by the N145S and D487N mutation and which had an overall lower HA titer and reduced affinity to guinea pig and human erythrocytes in comparison to the other tested viruses.

In conclusion, PSQ and cycle sequencing analyses revealed that the virus subgroup which is represented by the novel vaccine strain A/Victoria/361/2011 was the predominant A(H3N2) variant in 2011-2012. Interestingly, A(H3N2) viruses that did not agglutinate red blood cells predominated in the A/England/259/2011 subgroup indicating altered receptor specificities of those viruses.

Key words: influenza A(H3N2), characterization, pyrosequencing

P115

Specific amino acid in NS1 of highly pathogenic avian influenza A viruses effect the viral replication in mammalian cell.

Pumaree Kanrai*, Matthias Riedl and Stephan Pleschka

Institute of Medical Virology, Justus-Liebig-University Giessen, Schubertstrasse 81, D-35392 Giessen, Germany

Highly pathogenic avian influenza viruses (HPAIV) with reassorted NS segments from the HPAIV strains A/Goose/Guangdong/1/1996 (GD, H5N1) and A/Mallard/NL/12/2000 (Ma, H7N3) placed in the genetic background of HAPIV A/FPV/Rostock/34 (FPV-H7N1) were generated by reverse genetics. In mammalian cell culture systems the FPV-GD-NS reassortant shows increased replication ability including alteration of the polymerase activity compared to the wt FPV, while the FPV-Ma-NS is strongly impaired. Nevertheless, in avian cell culture systems both reassortant viruses as well as the wild type FPV replicate similar. The NS-genes of GD and Ma only differ in 8 amino acids (aa) in the NS1- and 2 aa in the NS2/NEP protein. Consequently, we are analyzing which aa or combination of aa in the GD-NS1 would be responsible for the enhanced replication of the FPV-GD-NS virus in mammalian cells. Therefore, we employ mutational analysis and generation of recombinant reassortant viruses with Ma-NS segments altered towards the GD-NS sequence to examine several aspects of viral replication, such as (i) viral propagation/growth rate and transmission/host range, (ii) an effect on the viral polymerase regarding replication, transcription and the export of viral genome and (iii) effects of the altered NS1 proteins on the innate immunity and cell-mediated apoptosis. The analysis of recombinant reassortants in mammalian and avian cell culture systems shows that specific changes in the aa-sequence of the Ma-NS segment indeed increase the replication ability of the recombinant reassortant virus in mammalian cell culture, but interestingly none of the changes increased the replication of the different recombinant reassortants in avian cell culture. Also, the differences between the NS2/NEP sequence is not responsible for the observed effects. The results so far indicate no selection advantage of the NS1 sequence differences in the FPV-GD-NS virus in avian cells and probably they will therefore not be selected, even though in mammalians systems they might be advantageous.

Key words: Influenza virus, host range

P116

Influenza virus infection of dendritic cells interferes with the immune response

Annette B. Vogel[1*], Lothar Stitz[1]

[1] Friedrich-Loeffler-Institute, Institute of Immunology, Germany

Influenza virus infection is a major topic in human health. Seasonal influenza virus infections cause a respiratory disease mostly in risk groups like infants, elderly and immunocompromised people. In contrast, highly pathogenic avian influenza A viruses (HPAIV) cause severe disease in humans and could be the source for the next pandemic influenza. The basis for their high pathogenic potential is still not fully understood. Various investigations suggest that these viruses interfere with the immune system leading to hypercytokinemia and reduction of the lymphocyte population. In addition, the presence of cytokines and chemokines in the lung of infected individuals results in an uncontrolled influx of immune mediators and consequently to a massive lung damage. Moreover, some influenza virus subtypes like HPAIV infect dendritic cells. This cell type could represent a very efficient vehicle for the spread of virus into lymphoid organs. Importantly, dendritic cells (DCs) play a crucial role in activating the adaptive immune response and an imbalance of activation signals may lead to a lack of adaptive immune response activation. As DCs are capable of replicating virus, we hypothesized that influenza virus infection interferes with cellular mechanisms resulting in an inappropriate immune response. Early factors are the activation of e.g. Interferon-regulatory-factors. First results revealed that DCs respond differently to common activation agents compared to other cell lines. Moreover, different influenza virus subtypes show altered activation and translocation structures for important early factors in the immune response. We conclude that IAV subtypes causing severe disease interfere with DC function.

Key words: dendritic cell

P117

Characterization of 7:1 gene reassortants of H1N1/09 pandemic influenza virus containing different N1 neuraminidases

T. Gerlach[1*]; L. Kühling [1]; T. Matrosovich[1]; F. Schwalm[1]; H.-D. Klenk[1]; M. Matrosovich[1]

[1]Institute of Virology, Philillpps University, Hans-Meerwein-Str. 2, D-35043 Marburg, Germany

The 2009 pandemic H1N1 influenza virus (H1N1pdm) is a reassortant virus containing the HA gene from a North American swine virus and the NA gene from a Eurasian avian-like swine virus. To assess the importance of this specific HA/NA constellation for the emergence of H1N1pdm, we generated ten 7:1 recombinant viruses that shared seven genes of A/Hamburg/5/09 and contained the N1 NA gene from representative avian, swine and seasonal human viruses. Seven reassortant viruses with heterologous NAs from avian, swine and human viruses eluted more slowly from red blood cells, replicated less efficiently in laboratory cells and were more sensitive to neutralization by human airway mucins as compared with H1N1pdm and viruses containing the NAs of the Eurasian avian-like swine virus A/swine/England/195682/92 and the triple reassortant North American swine virus A/Ohio/2/07. This pattern correlated with the efficiency of virus infection in differentiated cultures of human airway epithelial cells. We conclude that the origin of the NA gene can have a profound effect on virus sensitivity to airway mucins and replication in human airway epithelium and that functional properties of the NA of the H1N1pdm were optimal in this respect.

Key words: influenza, pandemic, neuraminidase, HA/NA balance, human airway mucins

P118

MAT1 and epitheliasin are HA-activating proteases of the influenza mouse model

C. Tarnow[1*], T. Steinmetzer[2], H. D. Klenk[1], W. Garten[1], E. Böttcher-Friebertshäuser[1]

[1]Institut für Virologie, Philipps-Universität Marburg, Hans-Meerwein-Straße 2, D-35043 Marburg
[2]Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marbacher Weg 6-10, D-35032 Marburg

Human influenza A viruses cause an acute infection of the respiratory tract that affects millions of people worldwide during annual outbreaks and occasional pandemics. Proteolytic activation of the influenza virus surface glycoprotein hemagglutinin (HA) by cellular proteases is thereby essential for viral infectivity and spread. Therefore, targeting of HA-activating proteases may provide an option to prevent influenza virus propagation. We identified the trypsin-like serine proteases HAT and TMPRSS2 from human airway epithelium as HA-activating proteases of influenza viruses with a monobasic cleavage site.
The mouse is the most frequently used model to study influenza virus replication in mammals. We identified the HAT- and TMPRSS2-homologous proteases MAT1 and epitheliasin which are expressed in murine respiratory tissue as proteases that are able to cleave and support proteolytic activation of influenza virus HA with a monobasic cleavage site. Multicycle replication in MAT1- or epitheliasin-expressing cells and murine tracheal organ culturs was efficiently suppressed by usage of specific peptide mimetic protease inhibitors. The inhibitory effect of the peptidomimetics was further enhanced when used in combination with common antivirals, such as oseltamivir.

Key words: hemagglutinin, proteolytic activation, serine protease inhibitors, animal model

P119

Data Curation of the GISAID EpiFlu Influenza Database

Anne Pohlmann[1*], Dirk Höper[1], Joachim Büch[2], Georg Friedrich[2], Thomas Lengauer[2], Jan Pohlmann[3], Martin Beer[1]

[1]Friedrich-Loeffler-Institut (FLI), Greifswald - Insel Riems, Germany; [2]Max Planck Institute for Informatics (MPII), Saarbrücken, Germany; [3]Federal Office for Agriculture and Food (BLE), Bonn, Germany

GISAID's EpiFlu database is the world's most comprehensive collection of influenza sequence data and corresponding epidemiological and clinical information. As of June 15th 2012 the database contains 250,218 sequences from 75,452 isolates. The quality of these data is crucial for any detailed analyses of datasets. Therefore systematic and scalable procedures for data curation are essential.

The Federal Republic of Germany is the host of the EpiFlu™ database. Three German institutions are engaged in development and maintenance of the database: the Max Planck Institute for Informatics (MPII) develops the software, the Federal Office for Agriculture and Food (BLE) hosts the GISAID portal, and the Friedrich-Loeffler-Institut (FLI) is responsible for quality control and data curation.

GISAID data curation comprises a two-stage process with automatic and manual annotation steps. During submission, the sequence is checked via an automatic procedure for correct annotation. The assignment of open reading frames and an examination for completeness are also facilitated by an automatic process. After release, data are manually inspected in a second curation phase. The metadata of each submission are checked for completeness and plausibility. The data curator also monitors the availability of sequences and is responsible for the rapid and continuous import of data from other databases. The latter requires extensive adaptation of data format and supplementation of lacking information.

In the future, GISAID will improve data analysis by connecting analysis software directly to the database. Established software tools will be made available from the database and thus simplify data analysis.

Key words: database, curation; metadata, data analysis, data quality

P120

Pandemic influenza A viruses escape from restriction by human MxA through adaptive mutations in the nucleoprotein

Dornfeld D.[1*], Mänz B.[1], Zimmermann P.[2],Haller O.[1], Kochs G.[1], SchwemmleM.[1]

[1]Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, 79104, Germany; [2]Institute of Medical Virology, University of Zurich, 8057 Zürich, Switzerland

The interferon induced human MxA protein is a potent restriction factor against avian influenza A virus infections. We therefore predicted that zoonotic transmission of these viruses into the human population is accompanied by the acquisition of adaptive mutations allowing evasion from MxA restriction. Here, we identified the MxA-adaptive mutations in the nucleoprotein (NP) of the pandemic strains A/Brevig Mission/1/1918(H1N1) (1918) and A/Hamburg/4/2009(H1N1) (pH1N1). Intriguingly, the amino acids (aa) conferring resistance towards MxA differ in both strains, but cluster to the same area of the body domain of NP. Sequence analysis revealed that the aa cluster required for MxA resistance in the 1918 strain remained highly conserved in all descendant seasonal and pandemic strains. However, the aa cluster in NP of the pH1N1 strain contains three unprecedented adaptive mutations, indicating an independent evolution of MxA resistance in this porcine-derived virus. Introduction of either the 1918 or the pH1N1 aa cluster into NP of an H5N1 virus mediated resistance to MxA but also decreased viral fitness. Vice versa, mutation of the corresponding aa cluster in pH1N1 NP to avian signature impaired MxA resistance, while viral growth was increased.
Taken together, the acquisitions of NP mutations required for MxA resistance emerged in both the 1918 and the 2009 pandemic strain independently and were most likely accompanied by compensatory mutations to overcome the associated strong attenuation.

Key words: MxA, adaptive mutations, influenza A virus nucleoprotein

P121

Elucidating the role of nucleoprotein in influenza A virus replication and transcription

L. Turrell*, E. Fodor, F. Vreede

Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE

The RNA genome of influenza A virus consists of eight single stranded negative sense vRNA segments. The vRNA segments are bound by viral RNA polymerase at the partially complementary 5' and 3' ends and associated with nucleoprotein (NP) to form a viral ribonucleoprotein (vRNP) complex. These vRNP complexes carry out both viral transcription and replication within the nucleus of the host cell. NP can self-oligomerise through a flexible tail loop that was identified in the crystal structure of NP. Mutations inhibiting self-oligomerisation of NP abrogate transcription and replication of genomic vRNA by the viral polymerase, however the role of NP in viral transcription and replication, although essential, is not well understood.

Here we show that the viral polymerase does not require NP for *in vivo* transcription and replication of short viral RNA (vRNA)-like segments up to approximately 100 nucleotides in length. vRNA-like templates longer than 100 nucleotides could not be replicated in the absence of NP. However, an oligomerisation mutant of NP was sufficient to support viral replication and transcription of intermediate length templates up to approximately 250 nucleotides in length. These findings support a role for NP in the elongation phase of viral transcription and replication but suggest that NP does not play a role in the regulation of initiation or termination of replication and transcription by the viral RNA polymerase.

Key words: nucleoprotein, oligomerisation, viral transcription and replication

P122

Cytokine expression at different stages of infection of A(H1N1)pdm09 in porcine lung using laser capture microdissection

D. Hicks, A. Orlowska, M. Kelly, B. Londt*, F. Garcon, S. M. Brookes and A. Núñez*

Animal Health and Veterinary Laboratories Agency - Weybridge

One of the features of influenza histopathology in the lung is the simultaneous presence of pulmonary lesions at different stages of infection and pathogenic development. Determination of the cytokine expression using tissue homogenates may mask opposing regulatory changes. For this reason, using pig as a host reservoir and a model for human infection with A(H1N1)pdm09 virus in which the disease is usually self limiting, we used laser capture microdissection (LCM) to identify pulmonary lobules containing lesions at different stages of infection and severity of inflammatory changes and excise them for further downstream analysis.

Pulmonary samples from pigs challenged with A/Hamburg/5/09 H1N1 virus were collected and snap-frozen for cryosectioning. The presence of viral antigen and histopathological changes were evaluated in individual lobules and classified using four different development stages corresponding to I-early infection, II-early inflammation, III-consolidation and IV-post infection. Individual lobules were visualised, excised using LCM and analysed by qRT-PCR for IL-8, IL-17a, TNF-α, IFN-γ, IL-4 and IL-10.

IFN-γ mRNA was down regulated during the early stages of disease pathogenesis coinciding with the highest abundance of virus. IL-8 and IL-10 was down regulated during early infection and early inflammation but upregulated during the consolidation and recovery stages, whereas IL-17a expression was upregulated throughout. No significant levels of IL-4 and TNF-α were detected.

This study highlights the value of LCM to evaluate the role of cytokines in the pathogenesis of pulmonary lesions in influenza infection.

Key words: H1N1 pandemic, pig model, laser capture microdissection, cytokines

P123

Promoter binding studies of the influenza A RNA polymerase

N.C. Robb[1*], A.I. Tomescu[1], N. Hengrung[2], E. Fodor[2] and A.N. Kapanidis[1]

[1]Biological Physics, University of Oxford, Parks Road, Oxford, UK
[2]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, UK

The influenza A virus genome consists of eight segments of negative-sense viral RNA (vRNA) with highly conserved, partially complementary termini. These termini are thought to adopt a corkscrew structure which, when bound by the influenza RNA polymerase, acts as a promoter for transcription and/or replication. Current models suggest that transcription (formation of a capped and polyadenylated mRNA) requires a single polymerase bound to both the 5' and 3' ends of the same vRNA template, while replication (formation of a full-length, non-capped and non-polyadenylated cRNA template) may be performed by multiple polymerases bound to a linear form of vRNA. We have used a combination of classical biochemistry techniques and a single-molecule protein induced fluorescence enhancement (PIFE) assay, in order to investigate binding of purified polymerase to the 5' and 3' ends of the promoter and further clarify the suggested models. We have obtained information on the binding constants and preliminary kinetics of the interactions and aim to investigate each step of transcription and replication initiation in real-time using novel single-molecule techniques.

Key words: polymerase, promoter, single-molecule

P124

Novel antiviral drug ingavirin^{R} restores the cellular antiviral response in influenza A virus infection and enhances viral clearance in ferrets

Irina Kuznetsova 1, Andrei Egorov 1, Thomas Aschacher 1, Vladimir Nibolsin 2, Michael Bergmann* 1

[1] Department of Surgery, Medical University of Vienna, Vienna, Austria;
[2] JSC Valenta Pharmaceuticals, Moscow, Russia

All licensed antiviral drugs against influenza A virus infection target structural proteins of the virus, allowing development of drug resistance by means of compensatory mutations. Another strategy to fight viral infections is the reinforcement of the antiviral cellular pathways, which are down-regulated by viral pathogenicity factors. Those pathways include activation of PKR, the translocation of IRF3 and IRF7 into the nucleus and the induction of MxA.

We here investigated the possible mechanism of action of a novel antiviral drug Ingavirin^{R} (Imidazolyl Ethanamide Pentandioic Acid), which is applied for upper respiratorial tract viral infections in Russia. We found, that infection of A549 lung epithelial cells with the wild type influenza A virus in the presence of the Ingavirin induces PKR activation, translocation of IRF3 and IRF7 and increases levels of MxA. Thus, wild type infection in the presence of the drug is associated with the induction of danger signal associated pathways, which are usually inhibited by the influenza virus NS1 protein.

We then tested the antiviral effect of the drug in ferrets. Ingavirin^{R} was given once per day in a dose of 13 mg/kg and therapy was started 36 hours after infection with influenza A(H1N1)pdm09 virus. Ingavirin^{R} accelerated viral clearance from nasal washes starting at day 4. No toxic side effects were observed.

We conclude that the drug may restore evolutionary evolved antiviral response pathways usually induced by pathogen associated patterns but inhibited by non-structural proteins of viruses.

Key words: antiviral drug, PKR, IRF-3, MxA, ferrets

P125

H5N1 virus in Russia (2005-2011): molecular epidemiology, ecology and evolution

Alexander Shestopalov*, Kirill Sharshov, Vasiliy Marchenko, Ivan Susloparov, Alexander Alekseev, Nikia Silko, Alexander Durymanov

Zoonotic Diseases and Influenza Department,
State Research Centre of Virology and Biotechnology «VECTOR », Novosibirsk, Russia

The report focuses on some epidemiological, ecological and evolutionary aspects of H5N1 virus in Russia from the first detection till now. The first influenza A (H5N1) outbreak in Russia was reported in the summer of 2005 in the territory of Western Siberia. The first case of Fujian sub-clade 2.3.2 influenza virus (H5N1) lineage in the Russian Far East was recorded in April 2008. Prior to this, no HPAI H5N1 outbreaks of the Fujian lineage had been reported in Russia. In June 2009, an outbreak of HPAI was recorded in wild birds in Mongolia and on the Uvs-Nuur Lake in Russia. Phylogenetic analysis of HA gene showed that viruses belong to clade 2.3.2. We hypothesized that bodies of water like the Qinghai Lake and the Uvs Nuur Lake may play an important role in the circulation of avian influenza so we suggested enhancing of surveillance program in this area and therefore we continued to study new outbreaks thoroughly (Sharshov et al., 2010). Our hypothesis was confirmed in June 2010. An outbreak of HPAI was recorded in wild birds at the Uvs Nuur Lake (OIE, 2010). Phylogenetic analysis of the hemagglutinin gene showed a close relation to strains isolated during outbreaks at the same location in 2009, at the Qinghai Lake in 2009 and in Mongolia in 2010 as all of them fall into clade 2.3.2. We can suppose that these strains originally appeared before or around 2009 at the Qinghai Lake and later in May 2010 caused an outbreak in Central Mongolia (Sakoda et al., 2010). In June 2010 dead birds were found at the Uvs Nuur Lake and viruses with 98-99% identity to Mongolian strains were isolated. The report contains detailed comparative virological, molecular, pathogenic characteristic H5N1viruses isolated in Russia (2005-2011).

Key words: H5N1, clade 2.3.2, Mongolia, waterbodies

P126

Analysis of the receptor binding properties of influenza A hemagglutinins using soluble chimeric proteins

Anne-Kathrin Sauer[1*], Chi-Hui Liang[2], Maren Bohm[1], Christel Schwegmann-Weßels[1], Georg Herrler[1]

[1] Institute of Virology, University of Veterinay Medicine Hannover, [2] Genomics Research Center, Academia Sinica, Taipei, Taiwan

The influenza hemagglutinin is the viral surface glycoprotein responsible for receptor recognition and plays therefore an important part in the adaptation process. The receptor determinants for influenza viruses are terminal N-acetyl-neuraminic acids on glycan chains of glyoproteins and glycolipids. In the avian respiratory and intestinal tract these sialic acids are linked in alpha2,3 conformation to an underlying galactose whereas in the human respiratory tract alpha2,6-linkages are predominant. To differenciate between both linkage types, in many studies two plant lectins are utilized: the *Maackia amurensis* agglutinin (MAA) for alpha2,3-linked sialic acids and the *Sambucus nigra* agglutinin (SNA) for alpha2,6-linkages. As the binding properties of those lectins are affected by underlying sugars, we use soluble chimeric hemagglutinins to investigate the availability of receptor type sialic acids on cell surfaces and tissue sections.
Soluble hemagglutinins of the H7 and the H9 subtype have been tested in detail on different cell lines and tissue sections from avian trachea and the porcine respiratory tract. In addition we also have performed binding tests with an H5 and different H1 hemagglutinins of human and porcine origin to further investigate the presence of receptor type sialic acids.
So far, these analyses have shown that soluble hemagglutinins are a valuable tool to investigate the receptor binding properties of influenza viruses.

Key words: Hemagglutinin, sialic acids

P127

Identification of cellular proteins interacting with influenza A virus polymerase using a virus-based split-luciferase complementation assay

C. Chiang[1*], S. Munier[1], Y. Jacob[2], N. Naffakh[1]

[1]Institut Pasteur, Unité de Génétique Moléculaire des Virus à ARN, Département de Virologie, F-75015 Paris, France; [2] Institut Pasteur, Unité de Génétique, Papillomavirus et Cancer Humain, Département de Virologie, F-75015 Paris, France;

The polymerase of influenza A viruses, an heterotrimeric PB1-PB2-PA complex, ensures transcription and replication of the viral genome in the nucleus of infected cells. Its association with host factors occurs at various stages in the viral life cycle and is thought to contribute to pathogenicity and host adaptation. Here a virus-based split-luciferase complementation assay was used to identify cellular proteins interacting with the viral polymerase. Influenza A/WSN/33 viruses whose PB2, PB1, or PA subunits were fused at their C-terminal end to the Gluc1 or Gluc2 complementation fragment of *Gaussia princeps* luciferase were used to infect 293T cells that transiently expressed recombinant proteins fused to Gluc2 or Gluc1, respectively. Twenty-six cellular proteins that we had previously identified as potential interactors of the viral polymerase in a yeast-two-hybrid screen were included in these experiments. Luciferase activity resulting from the trans-complementation of Gluc1 and Gluc2 was measured at 6 hours post infection. As revealed by high luciferase activity levels, several cellular proteins showed direct interaction with the viral polymerase. The impact of Gluc1 or Gluc2 fusion at the C- or N-terminus for the set of cellular proteins will be discussed. While the functional significance of these interactions remains to be elucidated, our data document the potential of a convenient complementation assay to detect direct interactions between the viral polymerase and host proteins in an infectious context.

Key words: influenza virus, polymerase, virus-host cell interactions, luciferase complementation assay

P128

Human importin-α7 increases the half-life of the influenza virus polymerase subunit PB2

S. Thiele[1*] & G. Gabriel[1]

[1]Heinrich-Pette-Institute – Leibniz-Institute for Experimental Virology, Hamburg, Germany

It has been previously shown that highly pathogenic avian influenza viruses undergo a switch from importin-α3 to -α7 dependency upon adaptation to the mammalian host. In addition to their role as nuclear import factors, importin-α isoforms have been proposed to possess functions beyond nuclear transport, such as affecting viral RNA replication itself, their involvement in vRNP assembly as well as host specific regulation of the viral polymerase activity.
Furthermore, for non-classical importin-β related nuclear transport factors it has been shown that they can fulfil a dual function, namely nuclear import and chaperone activity of exposed basic domains.
In this study, we have analysed whether human importin-α isoforms may also affect the stability of interacting viral proteins. We found that the importin-α7 isoform specifically increases the half-life of the viral polymerase subunit PB2 derived from a mammalian influenza virus, while other importin-α isoforms (α1, α3, α4 and α5) have no effect.
Currently, we are mapping the domains on the viral PB2 subunit and the importin-α7 isoform responsible for increasing the PB2 protein half-life.
Taken together, our data suggest that importin-α isoforms might be involved in protein stabilisation similar to importin-β related nuclear transport factors. Furthermore, the stabilisation of PB2 by the human importin-α7 isoform might play an additional role in influenza virus adaptation to the mammalian host.

Key words: influenza virus, importin-α, polymerase, stability, half-life

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A novel optical microscopy approach to characterize size and morphology of single influenza virus particles

E.C. Plötz[1], D.W. Siegersma[1], A.M. van Oijen[1], E. Fodor[2], N.C. Robb[2], & T. Cordes[1*]

[1]Molecular Microscopy Research Group & Single-molecule Biophysics, Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands; [2]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

We present the design and first experimental steps towards virus PAINT (vPAINT), a novel optical microscopy technique to visualize individual viral particles. We aim to use optical super-resolution microscopy with a spatial resolution of 5-10 nm to create images of virus particles in a manner that provides information on the spatial distribution of specific protein and lipid components.
vPAINT uses nanometric localization of single fluorescent molecules inside individual virus particles to reconstruct high-resolution images of the particle structure. Optical vPAINT images of influenza viruses give access to their size, morphology, distribution of surface proteins, and the arrangement of proteins within the virus particle itself. Thus, vPAINT has the potential to complement higher-resolution electron-microscopy (EM) techniques by providing optical images of viruses with 5-10 nm resolution under live-cell-compatible conditions without the need for staining, freezing or sectioning. We further envision visualization of dynamic processes, inaccessible to EM, such as virus assembly after replication in the host cell, as well as clinical diagnostic applications of vPAINT, i.e., identification of viral strains isolated from patients.
We expect that vPAINT will provide an exciting new optical toolkit for (dynamic) structural analysis of virus particles and correlation with functional properties such as single-particle fusion kinetics.

Key words: influenza structure, morphology, optical imaging, super-resolution microscopy, single-particle techniques

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Annexin A6 is a novel host factor in influenza A virus infection

A. Musiol[1*], S. Gran[1*], C. Erhardt[2], S. Ludwig[2], V.Gerke[1], U. Rescher[1]

[1] Institute of Med. Biochemistry (IMB), Center of Molecular Biology of Inflammation (ZMBE), University of Muenster;
[2] Institute of Molecular Virology (IMV), Center of Molecular Biology of Inflammation (ZMBE), University of Muenster

Host cell cholesterol is a critical factor in influenza A virus (IAV) replication; it is known that viral assembly, budding as well as infectivity are strongly dependent on cellular cholesterol distribution. However, molecular mechanisms of these interactions are largely unknown. Recently, Annexin A6 (AnxA6) was proposed as a regulator of cholesterol homeostasis. High levels of AnxA6 have been shown to induce cholesterol accumulation in late endosomes, thereby reducing cholesterol levels in the Golgi and plasma membrane.
Here we show that impaired cholesterol egress in A549 cells containing high AnxA6 levels results in significantly reduced viral titers, while depletion of AnxA6 leads to an increase. This effect could be approved by using the A431 cell line, which completely lacks AnxA6, in comparison to AnxA6 overexpressing A431 cells, hence suggesting a regulatory role for AnxA6 in IAV replication. Pharmacological accumulation of late endosomal cholesterol by U18666A leads to a comparable decrease of IAV titers in A549 cells, whereas treatment of AnxA6 overexpressing cells shows no further effect on viral replication; furthermore, adding exogenous cholesterol to AnxA6 overexpressing cells restores infectious titers. These observations reflect the regulatory role of AnxA6 in cholesterol homeostasis. Hence, these data indicate a role of AnxA6 as a novel host factor in IAV infection by modulating cholesterol homeostasis in host cells.

Key words: Annexin, cholesterol, late endosomes, IAV

P131

A novel vector to clone influenza A viruses segments for the bi-directional, simultaneous generation of vRNA and mRNA using a CcdB selection marker and alternative cloning sites

Ahmed Mostafa[1*], Matthias Riedl[1] and Stephan Pleschka[1]
[1]Institute of Medical Virology, Justus-Liebig-University Giessen, Schubertstr. 81, D-35392 Giessen, Germany

Reverse genetic of influenza A virus is simply the rescue of infectious virus from transfected cDNA of the eight genomic RNA-segments of influenza A virus. For this, the viral RNA-segments are cloned as cDNA into special vectors that allow the simultaneous generation of a Pol1-driven vRNA-like transcript of the segment, as well as the Pol2-driven generation of the according mRNA leading to the expression of corresponding protein(s). One of the most critical points in cloning the viral segments into of such expression vectors is an effective selection of vectors that contain the cDNA. To this point, we constructed a bi-directional vector encoding the lethal ccdB gene as a negative selection marker flanked with two AarI restriction/cloning sites between the human Pol1-promoter and the murine Pol1-terminator based on the pHW2000 vector by Hoffmann and Webster. In E. coli, the ccd locus of the F plasmid codes for two gene products, CcdA and CcdB. The CcdB protein which has a longer half-life than CcdA interferes with the DNA-gyrase, while CcdA acts as a repressor of CcdB. Therefore expression of CcdB over CcdA leads to cell death. CcdB protein is lethal for all E. coli except the strain DB3.1 (containing gyrA462 mutation). Through this newly constructed cloning vector, we overcome the drawbacks of other available cloning vectors (pHW2000blue) encoding LacZ as a selection marker that can lead to confusing results and which contains poorly digested BsmB1 restriction sites. We established two complete reverse genetic system of A/chicken/SA/CP7/1998 (H9N2) and S-OIV A/Giessen/1/09 (H1N1) to study the impact of NS reassortment of S-OIV with HPAIV (H5-and H7-) and of LPAI (H9N2) on the S-OIV host range, replication efficiency and virus-induced cellular response.

Key words: Influenza virus, cloning vector, reassortment

PARTICIPANTS

A

Amorim, Maria
Instituto Gulbenkian de Ciencia
Portugal
mjamorim@igc.gulbenkian.pt

Anfimov, Pavel
Ministry of Health and Social Development,
Research Institute of Influenza
Saint-Petersburg, Russia
anfimov@influenza.spb.ru

Anhlan, Darisuren
University of Münster,
Institute of Molecular Virology
Germany
anhlan@uni-muenster.de

Arikainen, Artur
University of Cambridge
United Kingdom
aaa67@cam.ac.uk

Arilahti, Veera
National Institute for Health and Welfare
Finland
veera.arilahti@thl.fi

Arnold, Catherine
Tulane University School of Medicine,
Department of Microbiology & Immunology
United States
carnold6@tulane.edu

Asanuma, Hideki
National Institute of Infectious Diseases
Japan
asanuma@nih.go.jp

Asaridou, Chloi-Magdalini
University of Münster,
Institute of Molecular Virology
Germany
asaridou@uni-muenster.de

Ashcroft, Jonathan
Imperial College London
United Kingdom
j.ashcroft10@imperial.ac.uk

B

Bahgat, Mahmoud
Helmholtz Center for Infection Research,
Department of Infection Genetics
Germany
Mahmoud-Bahgat.Riad@helmholtz-hzi.de

Barnekow, Angelika
University of Münster,
Department of Expirimental Tumorbiology
Germany
barneko@uni-muenster.de

Baumann, Jan
jan.baumann@staff.uni-marburg.de
Philipps Universität Marburg
Germany

Becher, Anne
Charité Berlin
Germany
anne.becher@charite.de

Becker, Christin
University of Giessen
Germany
Christin.Becker@Innere.med.uni-giessen.de

Berg, Johanna
Charité Berlin
Germany
Johanna.Berg@charite.de

Bergmann, Michael
Medical University of Vienna,
Department of Surgery
Austria
michael.bergmann@meduniwien.ac.at

Bertram, Stephanie
German Primate Center
sbertram@dpz.eu
Germany

Bildanova, Evgenia
Ministry of Health and Social Development,
Research Institute of Influenza
Russian Federation
er.billdann@gmail.com

Bogs, Jessica
Friedrich-Loeffler-Institute
Greifswald-Insel Riems, Germany
jessica.bogs@fli.bund.de

Börgeling, Yvonne
University of Münster,
Institute of Molecular Virology
Germany
borgelin@uni-muenster.de

Böttcher-Friebertshäuser, Eva
Philipps University Marburg,
Institute of Virology
Germany
friebertshaeuser@staff.uni-marburg.de

Bradley, Ben
Tulane University School of Medicine
United States
bbradley@tulane.edu

Brett, Katharina
Freie Universität Berlin,
Fachbereich Veterinärmedizin,
Institut für Immunologie und
Molekularbiologie
Germany
katharina.brett@gmail.com

Brunotte, Linda
University of Freiburg
Germany
linda.brunotte@uniklinik-freiburg.de

Buda, Silke
Robert Koch Institute
Germany
BudaS@rki.de

Budt, Matthias
Robert Koch-Institute
Germany
budtm@rki.de

C

Carron, Coralie
Université Lyon
France
coracarron@gmail.com

Castrucci, Maria Rita
Istituto Superiore di Sanità
Rome, Italy
mariarita.castrucci@iss.it

Chan, Renee
University of Hong Kong
reneewy@hku.hk

Chang, Pengxiang
University of Nottingham,
School of Veterinary Medicine and Science
United Kingdom
changpengxiang@gmail.com

Chiang, Chiayn
Institut Pasteur
France
chiayn.chiang@pasteur.fr

Cordes, Thorben
University of Groningen,
Zernike Institute for Advanced Materials,
Molecular Microscopy Research Group &
Single-molecule Biophysics
Netherlands
t.m.cordes@rug.nl

Czudai-Matwich, Volker
Philipps Universität Marburg,
Institut für Virologie
Germany
czudai@staff.uni-marburg.de

D

Danilenko, Daria
Research Institute of Influenza
St. Petersburg, Russia
daria.baibus@gmail.com

de Haan, Xander
Utrecht University
Netherlands
c.a.m.dehaan@uu.nl

de Vries, Erik
Utrecht University,
Veterinary Faculty,
Virology divison
Netherlands
e.devries@uu.nl

de Vries, Maren
Freie Universität Berlin
Germany
maren.devries@gmail.com

Deeg, Christoph
University of Freiburg,
Department of Virology
Germany
christoph.deeg@uniklinik-freiburg.de

Deng, Tao
Chinese Academy of Medical Sicences,
Institute of Pathogen Biology
China
tao.deng@ipbcams.ac.cn

Deng, Lei
Gent Univerisity
Belgium
lei.deng@dmbr.ugent.be

Dengler, Leonie
Helmholtz Centre for Infection Research
Braunschweig, Germany
Leonie.dengler@helmholtz-hzi.de

Denisova, Oxana
University of Helsinki,
Institute for Molecular Medicine
Finland
oxana.denisova@helsinki.fi

Di Pietro, Andrea
San Raffaele Scientific Institute
Italy
dipietro.andrea@hsr.it

Diekmann, Marieke
TU Braunschweig,
Institut für Biotechnologie
Germany
mariekedieckmann@web.de

Dierkes, Rüdiger
Universität Münster,
Institut für Molekulare Virologie
Germany
r_dier01@uni-muenster.de

Dornfeld, Dominik
University of Freiburg,
Institute for Medical Microbiology and Hygiene,
Department of Virology
Germany
dominik.dornfeld@gmx.de

Dortmans, Jos
University Utrecht
Faculty of Veterinary Medicine,
Netherlands
j.c.f.m.dortmans1@uu.nl

Dudek, Sabine Eva
University of Münster,
Institut of Molecular Virology
Germany
Sabine.Dudek@uni-muenster.de

Duwe, Susanne
Robert Koch-Institute
Germany
DuweS@rki.de

Dürrwald, Ralf
IDT Biologika GmbH
Dessau-Roßlau, Germany
ralf.duerrwald@idt-biologika.de

Dzieciolowski, Julia
Justus-Liebig-Universität Giessen,
Institute of Medical Virology
Germany
julia.dzieciolowski@viro.med.uni-giessen.de

E

Edinger, Thomas
University of Zurich
Switzerland
edinger.thomas@virology.uzh.ch

Ehrhardt, Christina
University of Münster,
Institute of Molecular Virology
Germany
ehrhardc@uni-muenster.de

Elsayed, Ahmed
Justus-Liebig-University Giessen,
Institute of Medical Virology
Germany
Ahmed.M.Elsayed@bio.uni-giessen.de

F

Fodor, Ervin
University of Oxford,
Sir William Dunn School of Pathology
United Kingdom
ervin.fodor@path.ox.ac.uk

Fouchier, Ron
Erasmus Medical Center,
Department of Virology
Netherlands
r.fouchier@erasmusmc.nl

Frensing, Timo
Max-Planck-Institute Magdeburg
Germany
frensing@mpi-magdeburg.mpg.de

Freuer, Catharina
Philipps University Marburg,
Institute of Virology
Germany
c.freuer@gmx.de

Friesenhagen, Judith
Medical School Hannover
Germany
Friesenhagen.Judith@mh-hannover.de

G

Gabriel, Gülsah
Heinrich-Pette-Institut für Virologie
Hamburg, Germany
guelsah.gabriel@hpi.uni-hamburg.de

Garulli, Bruno
University of Rome "La Sapienza"
Italy
bruno.garulli@uniroma1.it

Gereke, Marcus
Helmholtz Zentrum für
Infektionsforschung
Braunschweig, Germany
marcus.gereke@helmholtz-hzi.de

Gerlach, Thomas
Philipps-University Marburg,
Institute for Virology
Germany
thommi.gerlach@talk21.com

Goulet, Marie Line
McGill University/Jewish General Hospital
Montreal, Canada
marieline.goulet@gmail.com

Gran, Sandra
University of Münster
Institut of Molecular Virology
Germany
sandra.gran@t-online.de

H

Haasbach, Emanuel
Friedrich Loeffler Institute,
Institute of Immunology
Tuebingen, Germany
emanuel.haasbach@fli.bund.de

Härtle, Sonja
Ludwig-Maximilians-Universität München
Germany
Sonja.Haertle@lmu.de

Halbherr, Stefan
Institute of Virology and
Immunoprophylaxis
Switzerland
stefan.halbherr@ivi.admin.ch

Hale, Ben
MRC-University of Glasgow,
Centre for Virus Research
United Kingdom
ben.hale@glasgow.ac.uk

Hatesuer, Bastian
Helmholtz Center for Infection Research
Braunschweig, Germany
bastian.hatesuer@helmholtz-hzi.de

Herold, Susanne
University of Giessen,
Lung Center Giessen
Germany
susanne.herold@innere.med.uni-giessen.de

Herrler, Georg
Stiftung Tierärztliche Hochschule
Hannover
Germany
georg.herrler@tiho-hannover.de

Hillesheim, Andrea
University of Münster,
Institute of Molecular Virology (IMV)
Germany
a_hill16@uni-muenster.de

Hippenstiel, Stefan
Charité - Universitätsmedizin Berlin
Department of Internal
Medicine/Infectious Diseases and
Pulmonary Medicine
Berlin, Germany
stefan.hippenstiel@charite.de

Hiscott, John
Vaccine & Gene Therapy Institute Florida
United States
jhiscott@vgtifl.org

Hocke, Andreas
Charité Berlin
Germany
andreas.hocke@charite.de

Höfer, Chris
Humboldt-Universität zu Berlin,
Department of Biology, Molecular
Biophysics
Germany
hoeferct@hu-berlin.de

Högner, Katrin
Uniklinikum Giessen,
Infektionslabor
Germany
katrin.hoegner@innere.med.uni-giessen.de

Hoffmann, Julia
Heinrich-Pette-Institut,
Leibniz Institute for Experimental Virology
Hamburg, Germany
julia.richter@hpi.uni-hamburg.de

Hohenadl, Christine
Baxter Innovations GmbH
Vienna, Austria
christine_hohenadl@baxter.com

Holzberg, Magdalena
Institut für Molekulare Virologie
Germany
m.holzberg@t-online.de

Honda, Ayae
Hosei University
Japan
ayhonda@hosei.ac.jp

Horie, Masayuki
University of Freiburg,
Institute for Medical Microbiology and
Hygiene,
Department of Virology
Germany
masayuki.horie@uniklinik-freiburg.de

Hrincius, Eike-Roman
University of Münster,
Institute of Molecular Virology (IMV)
Germany
eikehrincius@googlemail.com

Hudjetz, Ben
Heinrich-Pette-Institut,
Leibniz-Institut für Experimentelle
Virologie
Germany
benjamin.hudjetz@hpi.uni-hamburg.de

Hutchinson, Edward
University of Oxford
United Kingdom
edward.hutchinson@path.ox.ac.uk

I

Iorio, Anna Maria
University of Perugia
Italy
anna@unipg.it

Isel, Catherine
Le Centre national de la recherche scientifique (CNRS)
France
C.Isel@ibmc-cnrs.unistra.fr

J

Jansen, Friederike
Nationale Forschungsplattform für Zoonosen
c/o Universität Münster
Germany

Jiang, Miao
University of Helsinki,
National Institute for Health and Welfare,
Institute of Biotechnology
Finland
miao.jiang@helsinki.fi

Julkunen, Ilkka
University of Helsinki,
National Institute for Health and Welfare
Helsinki, Finland
ilkka.julkunen@thl.fi

K

Kainov, Denis
Institute for Molecular Medicine (FIMM)
Finland
Denis.kainov@helsinki.fi

Kallfass, Carsten
University of Freiburg
Germany
carsten.kallfass@uniklinik-freiburg.de

Kalthoff, Donata
Friedrich-Loeffler-Institut
Greifswald - Insel Riems, Germany
donata.kalthoff@fli.bund.de

Kanrai, Pumaree
Justus-Liebig-University Giessen,
Institute of Medical Virology
Germany
pumaree.kanrai@viro.med.uni-giessen.de

Karlas, Alexander
Max-Planck-Institute for Infection Biology
Berlin, Germany
karlas@mpiib-berlin.mpg.de

Karpinskaia, Liubov
Influenza Research Institute
St. Petersburg, Russia
lubakarp@gmail.com

Kaspari, Marion
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
Germany
marion.kaspari@bvl.bund.de

Kavid, Narges
Razi Vaccine and Serum Research Institute
Karaj, Iran
n.kavid@gmail.com

Kawaoka, Yoshihiro
University of Wisconsin,
School of Veterinary Medicine,
Department of Pathobiological Sciences
Madison, United States
kawaokay@svm.vetmed.wisc.edu

Khamis, Zahra
Ministry of Health
Bahrain
zkhamis@health.gov.bh

Kistner, Otfried
Biomedical Research Center
Austria
otfried_kistner@baxter.com

Klemm, Carolin
University of Münster,
Institute of Molecular Virology
Germany
klemmc@uni-muenster.de

Knepper, Jessica
Robert Koch-Institut
Berlin, Germany
KnepperJ@rki.de

Kochs, Georg
University of Freiburg,
Department of Virology
Germany
georg.kochs@uniklinik-freiburg.de

Köther, Katharina
University of Münster,
Institute of Molecular Virology (IMV)
Germany
k.koether@uni-muenster.de

Kollmus, Heike
Helmholtz Centre for Infection Research
Braunschweig, Germany
heike.kollmus@helmholtz-hzi.de

Koopman, Gerrit
Biomedical Primate Research Centre
Rijswijk, Netherlands
koopman@bprc.nl

Krafft, Amy
National Institutes of Health
United States
kraffta@niaid.nih.gov

Kuchipudi, Suresh
University of Nottingham
United Kingdom
suresh.kuchipudi@nottingham.ac.uk

Kuzmina, Irina
State Research Center of Virology and Biotechnology
Novosibirsk, Russia
irenakuz@hotmail.com

Kuznetsova, Irina
Medical Univsersity of Vienna
Austria
irina.kuznetsova@meduniwien.ac.at

L

Lange, Jeannette
University Hospital Jena,
Institute for Virology and Antiviral Therapy
Germany
Jeannette.Lange@med.uni-jena.de

Leibenko, Liudmyla
L.V. Gromashevsky Institute of Epidemiology & Infectious Diseases AMS of Ukraine,
Kiev, Ukraine
leiblu@mail.ru

Leitzinger, Christin
Universitätsklinikum Jena,
Institut für Virologie und Antivirale Therapie
Germany
Christin.Leitzinger@med.uni-jena.de

Lesch, Markus
Max Planck Institute for Infection Biology
Berlin, Germany
lesch@mpiib-berlin.mpg.de

Liedmann, Swantje
University of Münster,
Institute of Molecular Virology (IMV)
Germany
s.liedmann@gmx.net

Linster, Martin
Erasmus Medical Center
Rotterdam, Netherlands
linster@erasmusmc.nl

Liu, Xiaoling
Chinese Academy of Sciences
China
xiaolingliu03@163.com

Lohmeyer, Juergen
Justus-Liebig-University Giessen,
Department of Internal Medicine
Germany
juergen.lohmeyer@innere.med.uni-giessen.de

Londt, Brandon
Animal Health & Veterinary Laboratories Agency (AHVLA)
United Kingdom
brandon.londt@ahvla.gsi.gov.uk

Loregian, Arianna
University of Padova
Italy
arianna.loregian@unipd.it

Lu, Yinghui
Philipps-Universität-Marburg,
Institut für Virologie
Germany
jessie_lu@web.de

Ludersdorfer, Thomas
Swiss Federal Veterinary Office,
Institute of Virology and Immunoprophylaxis (IVI)
Mittelhäusern, Switzerland
ludersdorfer.thomas@gmail.com

Ludwig, Stephan
University of Münster,
Institute of Molecular Virology (IMV)
Germany
ludwigs@uni-muenster.de

M

Müthing, Johannes
University of Münster,
Institute for Hygiene
Germany
jm@uni-muenster.de

Mair, Caroline
Humboldt University of Berlin,
Molecular Biophysics
Germany
caroline.mair@hu-berlin.de

Mantovani, Giancarlo
University of Perugia
Italy
mantovani@pg.infn.it

Marquet, Roland
Le Centre national de la recherche scientifique (CNRS)
France
r.marquet@ibmc-cnrs.unistra.fr

Martínez Alonso, Mónica
University of Oxford
United Kingdom
monica.martinezalonso@path.ox.ac.uk

Masemann, Dörthe
University of Münster,
Institute for Moleculare Virology
Germany
d_mase01@uni-muenster.de

Matrosovich, Mikhail
University of Marburg,
Institute of Virology
Germany
M.Matrosovich@gmail.com

Matrosovich, Tatyana
University of Marburg,
Institute of Virology
Germany
T.Matrosovich@googlemail.com

Mbaika, Sophiah
Jomo Kenyatta University of Agriculture and Technology
Kenya
sophiambaika@yahoo.com

McCullers, Jon
St. Jude Children's Research HospitalDepartment of Infectious Diseases
Memphis, United States
jon.mccullers@stjude.org

Mehnert, Nora
Helmholtz Zentrum für Infektionsforschung
Braunschweig, Germany
nora.mehnert@helmholtz-hzi.de

Meisen, Iris
University of Münster,
Institute for Hygiene
Germany
meisen@uni-muenster.de

Meng, Fandan
University of Veterinary Medicine
Germany
mengfandan11111@hotmail.com

Mersmann, Jana
Technische Universität Braunschweig
Institut für Biotechnologie
Germany
j.mersmann@tu-bs.de

Meseko, Clement
National Veterinary Research Institute
Nigeria
cameseko@yahoo.com

Michaelis, Martin
University of Kent
United Kingdom
M.Michaelis@kent.ac.uk

Mirfakhar, Seyed Ali
Iran
ali_mr64@yahoo.com

Mizgerd, Joseph P.
Boston University,
School of Medicine,
Pulmonary Center
United States
jmizgerd@bu.edu

Mooij, Petra
Biomedical Primate Research Centre
Rijswijk, Netherlands
mooij@bprc.nl

Mormann, Michael
University of Münster,
Institute of Medical Physics and Biophysics
Germany
mmormann@uni-muenster.de

Musiol, Agnes Katharina
University of Münster,
Center for Molecular Biology of Inflammation,
Institute of Medical Biochemistry
Germany
Agnes.musiol@gmail.com

N

Nacken, Wolfgang
University of Münster
Germany
nacken@uni-muenster.de

Naesens, Lieve
KU Leuven,
Rega Institute for Medical Research
Belgium
lieve.naesens@rega.kuleuven.be

Nelli, Rahul
University of Nottingham
United Kingdom
rahulnelli@gmail.com

Niemann, Silke
Uniklinikum Münster,
Institut für Medizinische Mikrobiologie
Germany
Silke_Niemann@gmx.de

Nitzsche, Katja
Universität Münster,
Institut für Molekulare Virologie
Germany
katjanitzsche@web.de

Nordhoff, Carolin
University of Münster
Institute of Molecular Virology
Germany
carolin-nordhoff@t-online.de

Nürnberger, Cindy
University of Freiburg,
Department Virology
Germany
cindy.nuernberger@uniklinik-freiburg.de

O

Österlund, Pamela
National Institute for Health and Welfare
Finland
pamela.osterlund@thl.fi

Omoleye, Segun Oluwafolaranmi
Ministry Of Agriculture,
Veterinary Services Ondo State
Nigeria
omoleyefs@yahoo.co.uk

Ortin, Juan
National Centre of Biotechnology,
Department of Molecular and Cellular Biology
Madrid, Spain
jortin@cnb.csic.es

Otte, Anna
Heinrich-Pette-Institute,
Leibniz Institute for Experimental Virology
Hamburg, Germany
anna.otte@hpi.uni-hamburg.de

P

Paki, Katharina
Robert Koch-Institut
Germany
PakiK@rki.de

Parvin, Rokshana
University of Leipzig,
Faculty of Veterinary Medicine,
Institute of Virology
Germany
parvin.rokshana@vetmed.uni-leipzig.de

Paterson, Duncan
University of Oxford
United Kingdom
duncan.paterson@path.ox.ac.uk

Patzina, Corinna
Uniklinik Freiburg,
Department of Virology
Germany
corinna.patzina@uniklinik-freiburg.de

Pereira, Carina
University of Cambridge
United Kingdom
cfp28@cam.ac.uk

Pilzner, Carolin
Helmholtz Zentrum für Infektionsforschung
Braunschweig, Germany
cpi12@helmholtz-hzi.de

Pleschka, Stephan
Justus-Liebig-University Giessen,
Institute of Medical Virology
Germany
stephan.pleschka@mikro.bio.uni-giessen.de

Pohl, Marie
University of Zurich,
Institute for Medical Virology
Switzerland
pohl.marie-theres@gmail.com

Pohlentz, Gottfried
University of Münster,
Institute for Hygiene
Germany
pohlentz@uni-muenster.de

Pohlmann, Anne
Friedrich-Loeffler-Institut
Germany
anne.pohlmann@fli.bund.de

Prabakaran, Mookkan
Temasek Lifesciences Laboratory
Singapore
prabakar@tll.org.sg

Prokopeva, Elena
1 State Research Center of Virology and Biotechnology SRC VB "Vector"
Novosibirsk Region, Russia
ellap@live.ru

Q

Qiu, Yu
Ghent University
Faculty of Veterinary Medicine,
Laboratory of Virology
Belgium
yu.qiu@ugent.be

Quang, Trong-Hung
Universitätsklinikum Münster
Germany
tronghungquang@yahoo.de

Quantius, Jennifer
Justus-Liebig-Universität Giessen
Germany
Jennifer.Quantius@innere.med.uni-giessen.de

R

Radu, Cojocaru
National Centre for Public Health
Moldova
radu_cojocaru@hotmail.com

Resa-Infante, Patricia
Heinrich-Pette-Institut ,
Leibniz Institute for Experimental Virology
Hamburg, Germany
patriciaresainfante@gmail.com

Rethwill, Axel
Universität Würzburg,
Institut für Virologie
Germany
virologie@vim.uni-wuerzburg.de

Reuther, Peter
University of Freiburg,
Department of Virology
Germany
peter.reuther@uniklinik-freiburg.de

Richter, Martina
Universitätsklinkum Jena,
Institut für Virologie und Antivirale Therapie
Germany
martina.richter1@med.uni-jena.de

Robb, Nicole
University of Oxford
United Kingdom
n.robb1@physics.ox.ac.uk

Roche, Gerard
Imperial College London
United Kingdom
gerard.roche11@imperial.ac.uk

Röhrs, Susanne
Friedrich Loeffler Institut
Germany
susanne.roehrs@fli.bund.de

Roell, Susanne
Ludwig-Maximilians-Universität München,
Institute for Animal,
Department for Veterinary Sciences
Physiology
Germany
S.Roell@tiph.vetmed.uni-muenchen.de

Rosa-Calatrava, Manuel
Vircell-Virpath /UCBL-HCL
Lyon, France
manuel.rosa-calatrava@univ-lyon1.fr

Rossman, Jeremy
University of Kent
United Kingdom
j.s.rossman@kent.ac.uk

Rubbenstroth, Dennis
Department Virology,
University of Freiburg
Germany
Dennis.Rubbenstroth@uniklinik-freiburg.de

Rudowitz, Daniela
Elanco Animal Health
Lilly Deutschland GmbH
Bad Homburg, Germany
rudowitz_daniela@elanco.com

Rückle, Andrea
University of Münster,
Institute of Molecular Virology
Germany
andrea_rueckle@gmx.de

S

Sauer, Anne-Kathrin
University of Veterinary Medicine,
Institute for Virology
Hannover, Germany
anne-kathrin.sauer@tiho-hannover.de

Schierhorn, Kristina L.
Robert Koch-Institute
Germany
schierhornk@rki.de

Schilling, Mirjam
University of Freiburg,
Department of Virology
Germany
mirjamschilling@yahoo.de

Schmid, Isabell
Nationale Forschungsplattform für Zoonosen
c/o Universität Münster
Germany
isabell.schmid@ukmuenster.de

Schmidtke, Michaela
University Hospital Jena
Germany
michaela.schmidtke@med.uni-jena.de

Schmitt, Jutta
Regierungspräsidium Tübingen – Gentechnikaufsicht
Germany
jutta.schmitt@rpt.bwl.de

Schotsaert, Michael
VIB/DMBR/Ugent
Zwijnaarde, Belgium
michael.schotsaert@dmbr.ugent.be

Schughart, Klaus
Helmholtz-Zentrum für Infektionsforschung
Braunschweig, Germany
syw@helmholtz-hzi.de

Schwalm, Folker
Institut für Virologie der Universität Marburg
Germany
folker.schwalm@staff.uni-marburg.de

Schwemmle, Martin
Department of Virology,
University Freiburg
Germany
martin.schwemmle@uniklinik-freiburg.de

Sediri, Hanna
Philipps University Marburg,
Institute for Virology
Germany
sediri@staff.uni-marburg.de

Seidel, Nora
Universitätsklinikum Jena,
Institut für Virologie und Antivirale Therapie
Germany
nora.seidel@med.uni-jena.de

Selvakumar, Balachandar
Justus Liebig University Giessen
Germany
balachandar.selvakumar@innere.med.uni-giessen.com

Sherstkov, Evgeny
Novosibirsk State University
Russia
eash7@bk.ru

Shestopalov, Alexandr
1 State Research Center of Virology and Biotechnology SRC VB "Vector",
Department of zoonotic diseases and influenza
Novosibirsk Region, Russia
silkonyu@gmail.com

Shin, Dai-Lun
Helmholtz Centre for Infection Research
Germany
dls12@helmholtz-hzi.de

Shtro, Anna
Influenza Research Institute
Saint-Petersburg, Russia
shtro@influenza.spb.ru

Siche, Stefanie
Freie Universität Berlin,
Institut für Immunologie und Molekularbiologie
Germany
st.siche@gmail.com

Sieben, Christian
Humboldt-Universität zu Berlin
Germany
christian.sieben@biologie.hu-berlin.de

Silko, Nikita
1 State Research Center of Virology and Biotechnology SRC VB "Vector", Novosibirsk Region, Russia
silkonyu@gmail.com

Silva, Izabella
University of Münster
Germany
izabellathais@hotmail.com

Si-Tahar, Mustapha
National Institute of Health and Medical Research (INSERM),
U1100 unit
Paris, France
si-tahar@univ-tours.fr

Soubies, Sebastien
University of Freiburg,
Department of Virology
Germany
sebastien.soubies@uniklinik-freiburg.de

Spinu, Constantin
National Centre for Public Health
Moldova
cspinu@cnsp.md

Spinu, Igor
National Centre for Public Health
Moldova

Standlee, Courtney
Tulane University
New Orleans, United States
cstandle@tulane.edu

Stech, Juergen
Friedrich-Loeffler-Institut
Greifswald - Insel Riems,Germany
juergen.stech@fli.bund.de

Stegemann, Sabine
Helmholtz Zentrum für Infektionsforschung
Braunschweig, Germany
sabine.stegemann@helmholtz-hzi.de

Steglich, Constanze
Friedrich-Loeffler-Institute
Germany
Constanze.Steglich@fli.bund.de

Stela, Gheorghita
National Centre for Public Health
Moldova
sgheorghita@cnsp.md

Stertz, Silke
University of Zurich
Switzerland
stertz.silke@virology.uzh.ch

Stricker, Ruth
Helmholtz-Zentrum für Infektionsforschung
Braunschweig, Germany
ruth.stricker@helmholtz-HZI.de

Sugrue, Richard J.
Nanyang Technological University,
School of Biological Sciences,
Division of Molecular Genetics and Cell Biology,
Singapore
RJSugrue@ntu.edu.sg

T

Takashita, Emi
National Institute of Infectious Diseases
Tokyo, japan
emitaka@nih.go.jp

Tantawy, Mohamed
Helmholtz Centre for Infection Research
Braunschweig, Germany
dr_mohamedht@yahoo.com

Tarkhan-Mouravi, Olgha
National Center for Disease Control and Public Health of Georgia
Georgia
olgancdc@gmail.com

Tarnow, Carolin
Philipps Universität Marburg,
Institut für Virologie
Germany
tarnowc@staff.uni-marburg.de

te Velthuis, Aartjan
University of Oxford
United Kingdom
aartjan.tevelthuis@path.ox.ac.uk

Tenbusch, Matthias
Ruhr-University Bochum
Germany
matthias.tenbusch@rub.de

Terrier, Olivier
VirPath lab, VirCell Team
France
olivier.terrier@univ-lyon1.fr

Theisen, Linda
Centre de Recherche Public
Luxembourg
linda.theisen@crp-sante.lu

Thiele, Swantje
Heinrich-Pette-Institute,
Leibniz-Institute for Experimental Virology
Hamburg, Germany
swantje.thiele@hpi.uni-hamburg.de

Tomescu, Alexandra
University of Oxford
United Kingdom
a.tomescu1@physics.ox.ac.uk

Turrell, Lauren
University of Oxford
United Kingdom
laurenturrell@path.ox.ac.uk

V

van Boheemen, Sander
Erasmus MC
Rotterdam, Netherlands
s.vanboheemen@erasmusmc.nl

van den Hoecke, Silvie
VIB Department for Molecular Biomedical Research (DMBR)
Ghent, Belgium
silvie.vandenhoecke@dmbr.ugent.be

van der Vliet, Stefan
Erasmus MC,
Department of Virology
Rotterdam, Netherlands
s.vandervliet@erasmusmc.nl

Vanderlinden, Evelien
Rega Institute for Medical Research
Leuven, Belgium
evelien.vanderlinden@rega.kuleuven.be

Vasilyeva, Anastasiya
Research Institute of Influenza
Saint-Petersburg, Russia
nastukas@mail.ru

Veit, Michael
Free University Berlin
Germany
mveit@zedat.fu-berlin.de

Verhelst, Judith
DMBR/VIB/Ugent
Zwijnaarde, Belgium
judith.verhelst@dmbr.ugent.be

Vicenzi, Elisa
Ospedale San Raffaele
Milan, Italy
vicenzi.elisa@hsr.it

Vogel, Annette
Friedrich-Loeffler-Institute
Germany
Annette.Vogel@fli.bund.de

Voss, Thomas
Tulane University School of Medicine
New Orleans, United States
tvoss@tulane.edu

Voss, Kayleigh
Tulane University School of Medicine
New Orleans, United States
kayleigh_voss@yahoo.com

Vreede, Frank
University of Oxford
United Kingdom
frank.vreede@path.ox.ac.uk

W

Warnking, Kathrin
University of Münster
Institut of Molecular Virology
Germany
kathrin.warnking@uni-muenster.de

Wang, Zhenja
Free University of Berlin
Germany
zhenyawang@zedat.fu-berlin.de

Wash, Rachel
Wellcome Trust Sanger Institute
United Kingdom
Rc7@sanger.ac.uk

Wedde, Marianne
Robert Koch-Institute
Berlin, Germany
WeddeM@rki.de

Wendel, Isabel
Institute for Virology,
Philipps-University Marburg
Germany
isabel.wendel@staff.uni-marburg.de

Widjaja, Ivy
Utrecht University,
Faculty of Veterinary Medicine,
Division of Virology
Netherlands
i.widjaja@uu.nl

Wilk, Esther
Helmholtz-Zentrum für
Infektionsforschung
Braunschweig, Germany
esther.wilk@helmhlotz-hzi.de

Wise, Helen
University of Cambridge
United Kingdom
hw256@cam.ac.uk

Wixler, Viktor
University of Münster,
Institute of Molecular Virology (IMV)
Germany
vwixler@uni-muenster.de

Wolf, Meike
Goethe-University Frankfurt
Germany
meike.wolf@em.uni-frankfurt.de

Wolff, Thorsten
Robert Koch-Institute
Germany
wolfft@rki.de

Wurm, Rüdiger
Stadt Dortmund
Germany
dr.ruediger.wurm@stadtdo.de

X

Xu, Ke
Chinese Academy of Sciences,
Institut Pasteur of Shanghai
China
kxu@sibs.ac.cn

Y

York, Ashley
University of Oxford
United Kingdom
ashley.york@path.ox.ac.uk

Yver, Matthieu
Universität Lyon,
Virologie et Pathologie Humaine
France
matthieu.yver@univ-lyon1.fr

Z

Zakhashvili, Khatuna
National Center for Disease Control and
Public Health of Georgia
Georgia
episurv@ncdc.ge

Zarubaev, Vladimir
Influenza Research Institute
Saint-Petersburg, Russia
zarubaev@influenza.spb.ru

Zell, Roland
Universitätsklinikum Jena,
Institut für Virologie und Antivirale
Therapie
Germany
Roland.Zell@med.uni-jena.de

Ziehm, Dagmar
Niedersächsisches Landesgesundheitsamt
Germany
Dagmar.Ziehm@nlga.niedersachsen.de

Zimmer, Gert
Swiss Federal Veterinary Office,
Institute of Virology and
Immunoprophylaxis (IVI)
Mittelhäusern, Switzerland
gert.zimmer@ivi.admin.ch

Zirbes, Julian
Justus-Liebig-University Giessen
Germany
julian.Zirbes@med.uni-giessen.de

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