2014

Confirmed speakers

Michaela Gack | Boston, USA

George G. Gao | Beijing, China

Adolfo Garcia-Sastre | New York, USA

Paul Kellam | Cambridge, UK

Mauro Teixeira | Belo Horizonte, Brazil

4th International Influenza Meeting

FluResearchNet.

September 21–23

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

German Research Platform for Zoonoses

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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 21. Posters are to be removed between 14.00 and 16.30 p.m. on Tuesday, September 23.

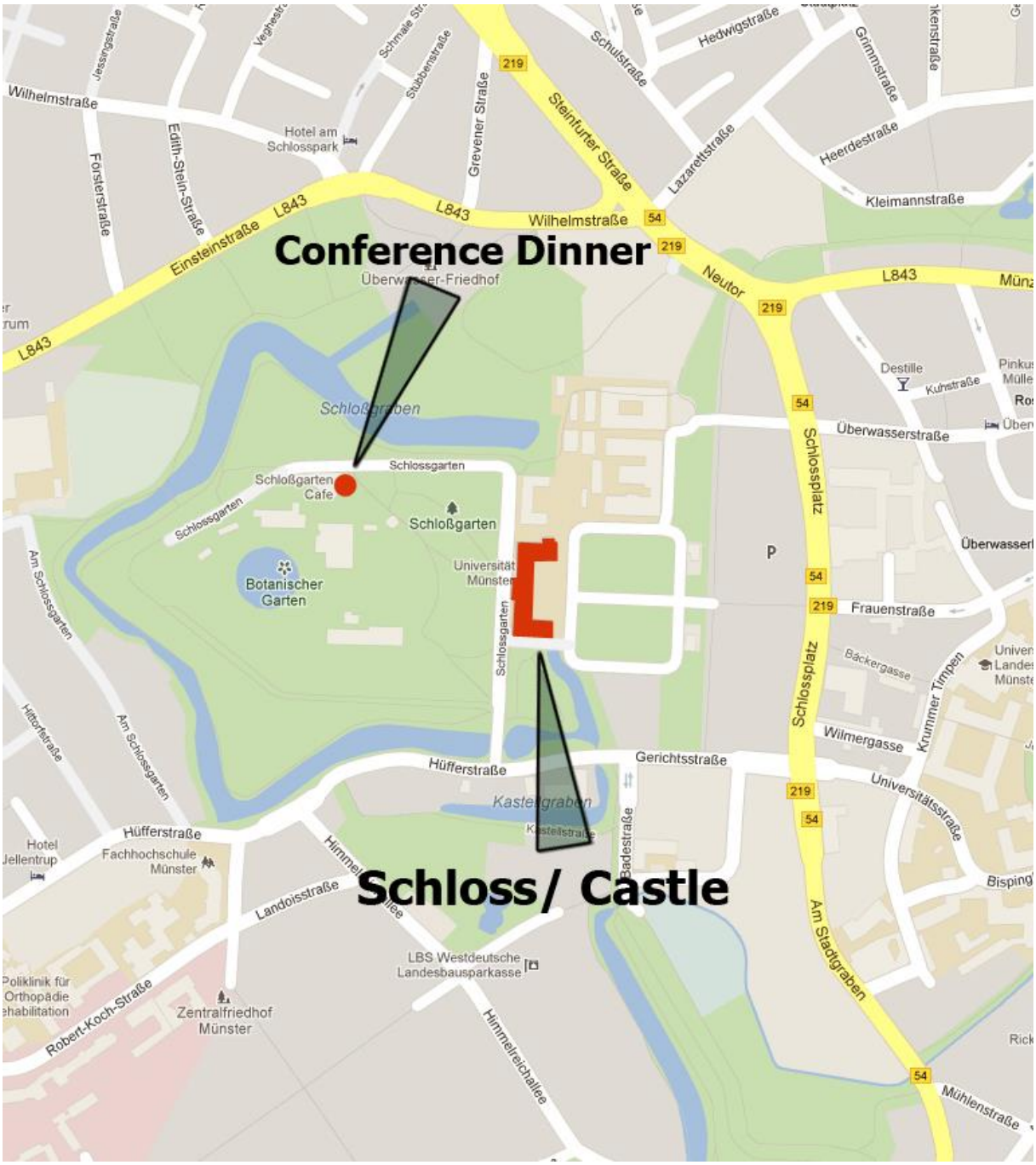
Meals
Lunches will be provided in the Foyer of the castle of Münster, as indicated in the program. Evening meals will be provided as part of the social program. You are invited by the organizers.

Social Program
The Opening reception will take place at the venue on Sunday, September 21 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 German Research 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.2014@uni-muenster.de

VENUE


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

Opening

Sunday, September 21 2014

Keynote lecture: Interactions of the influenza virus NS1 with the host

Adolfo Garcia-Sastre, New York, USA

Session 1

HOST GENETICS OF INFECTIONS

Chair: Peter Stäheli

Monday, September 22 2014

Keynote lecture: Virus and host genetics of infectious disease severity
Paul Kellam, Cambridge, United Kingdom

Genetic background influences the antiviral activity of Mx1 gene to influenza virus infection in mice
Dai-Lun Shin, Braunschweig, Germany

Severely reduced influenza A virus resistance of wild-derived CAST/EiJ mice due to two amino acid changes in the interferon-induced Mx1 resistance factor
Cindy Nürnberger, Freiburg, Germany

Analysis of the host response to influenza A virus infection in the Collaborative Cross founder strains
Sarah R. Leist, Braunschweig, Germany

Genetic background influences the antiviral activity of *Mx1* gene to influenza virus infection in mice

Dai-Lun Shin[1,2] ; Bastian Hatesuer[1] ; Klaus Schughart[1,2,3]

1 Helmholtz Centre for Infection Research, Germany
2 University of Veterinary Medicine Hannover, Germany
3 University of Tennessee, Health Science Center, United-State

It was shown previously that mice carrying a functional Myxovirus resistance genes 1 (*Mx1*) allele are highly resistant against IAV. It acts as a restriction factor for virus nuclear import and blocks viral replication. To date, the influence of genetic background and relationship with other genes were has not yet been studied. We showed previously that genetic background greatly influences the severity of disease after IAV infections when comparing between the laboratory inbred strains DBA/2J (D2) and C57BL/6J (B6). In this study, we used B6.A2G-*Mx1*$^{+/+}$ mice and A/Puerto Rico/8/34 H1N1 Freiburg variant virus (PR8F) and compared the phenotype with B6 mice which contain nonfunctional *Mx1* allele. Furthermore, we changed the genetic background for B6.A2G-*Mx1*$^{+/+}$ mice from B6 into D2 background via backcrossing with D2 for 10 generations. D2.A2G-*Mx1*$^{+/+}$ mice were highly susceptible to IAV infection. Mice with functional *Mx1* allele in D2 background exhibited 100% mortality after days 7 post infection. F1 mice from a cross of D2.A2G-*Mx1*$^{+/+}$ and B6 regained its antiviral activity against IAV infection. Our results suggest that B6 contain genetic factors which initiate *Mx1* antiviral function by restricting viral replication, which are absence in D2 mice.

Keywords: *Mx1* gene, mouse model, genetic background

Severely reduced influenza A virus resistance of wild-derived CAST/EiJ mice due to two amino acid changes in the interferon-induced Mx1 resistance factor

Cindy Nürnberger[1,2], Katrin Friedrich[1], Vanessa Zimmermann [1], Peter Staeheli[1]

[1] Institute for Virology, University Medical Center Freiburg
[2] Spemann Graduate School of Biology and Medicine (SGBM), Albert Ludwigs University Freiburg

Type I and III interferon-induced Mx genes encode evolutionary conserved potent antiviral restriction factors inhibiting a broad range of RNA viruses in most vertebrate species. In mice, the Mx1 locus confers a high degree of resistance to orthomyxoviruses, such as Influenza A (IAV) and Thogoto virus (THOV). Mice of the A2G strain carry a functional Mx1 gene. In contrast, most laboratory mouse strains carry defective Mx1 alleles and are highly susceptible to IAV and THOV challenge.

Recent work by others* showed that CAST/EiJ mice, an inbred strain derived from wild *Mus musculus castaneus,* are susceptible to IAV challenge, although they carry a full-length Mx1 gene. We were able to confirm the IAV susceptibility of mice carrying a CAST Mx1 allele. However, we noted that these animals resembled A2G mice by being resistant to other orthomyxoviruses, such as THOV and Dhori virus. We further noted that CAST-derived Mx1 protein is able to restrict influenza A virus polymerase activity to an intermediate extent, suggesting that this Mx1 allele is able to confer residual anti-influenza activity. In addition, residual activity of the CAST-derived Mx1 allele could be detected when mice were infected with H5N1 and H7N7 influenza viruses that are particularly susceptible to Mx protein restriction. Sequencing revealed that CAST-derived Mx1 differs by two amino acids from A2G-derived Mx1 which are localized in the G domain. To better understand Mx1-mediated viral restriction, we are currently investigating the individual contributions of these two amino acids to antiviral capacity and GTPase activity.

* Ferris et al., PLOS pathogens, 2013

Keywords: Influenza A virus restriction, Thogoto virus, interferon-induced innate immunity, antiviral Mx1, CAST/EiJ mice

Analysis of the host response to influenza A virus infection in the Collaborative Cross founder strains

Sarah Leist[1], Carolin Pilzner[1], Heike Kollmus[1] and Klaus Schughart[1,2,3]

[1]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[2]University of Veterinary Medicine Hannover, Germany
[3]University of Tennessee Health Science Center, Memphis, USA

Genetic reference populations (GRPs) of the mouse species are a sophisticated and suitable tool to investigate host factors that contribute to resistance or susceptibility to infections. Through tools like this the genetic diversity present in the human population can be simulated. The recently established Collaborative Cross (CC) is a GRP derived from eight genetically different founder strains. These founder strains were bred in a specific breeding funnel resulting in 500 – 700 CC lines, each of them representing a unique mosaic of the genetic information of the eight founder strains. CC lines are used for genetic mapping studies and are a well-suited method to investigate host factors that influence susceptibility and resistance to influenza A infections.
Here, we present our analysis of the eight CC founder strains (A/J, C57BL/6J, 129S1/SvlmJ, NOD/ShiLtJ, NZO/HILtJ, PWK/PhJ, CAST/EiJ and WSB/EiJ) and four CC lines (OR13140, OR13067, IL16188, IL16211) after infection with the mouse-adapted virus strain influenza A/HK/01/68 (H3N2). Female as well as male mice were infected intra-nasally with up to three different concentrations of H3N2. Body weight and survival was monitored for 14 days. In addition, we characterized the host response in more detail by analyzing hemogram of peripheral blood and determining viral loads in infected lungs. We observed large differences between the CC founder strains and the four CC. The five different *Mx1* alleles present in the CC founder strains influenced the infection outcome. However the analysis of the four CC lines showed that the function of Mx1 is modulated by the genetic background.

Keywords: Collaborative Cross, mouse, host pathogen interaction

Session 2

VIRUS HOST CELL INTERACTION

Chair: Stephan Ludwig

Monday, September 22 2014

Downregulation of the Na,K-ATPase in the alveolar epithelium after Influenza A infection is dependant on the kinases CaMKKb and AMPK
Christin Becker, Gießen, Germany

The tetraspanin proteins, CD81 and CD9, promote efficient influenza A virus budding through interactions with HA and NA
Yuhong Liang, New York, USA

Influenza Virus-induced Caspase-dependent Enlargement of Nuclear Pores Coincides with Nuclear Export of Viral Ribonucleoprotein Complexes
Stephan Pleschka, Gießen, Germany

Type I interferon signalling is inhibited upon influenza A virus and Staphylococcus aureus co-infection
Christina Ehrhardt, Münster, Germany

Downregulation of the Na,K-ATPase in the alveolar epithelium after Influenza A infection is dependant on the kinases CaMKKb and AMPK

Becker C, Morales-Nebreda L, Lecuona E, Wolff T, Vadasz I, Morty RE, Lohmeyer J, Seeger W, Sznajder JI, Mutlu G, Budinger GRS, and Herold S

Universities of Giessen and Marburg Lung Center (UGMLC), Member of the German Center for Lung Research (DZL), Giessen, Germany; Division of Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois; Robert Koch-Institut, Berlin, Germany; Max-Planck-Institute for Heart and Lung Research, Member of the German Center for Lung Research (DZL), Nauheim, Germany

Influenza A viruses (IV) can cause primary viral pneumonia resulting in acute respiratory distress syndrome (ARDS) associated with edema formation. Clearance of excessive water from the alveolar airspace is driven by epithelial Na,K-ATPase, therefore we investigated the regulation of Na,K-ATPase and the effect on alveolar fluid clearance (AFC) in the lung epithelium after IV-infection.
Gene expression levels of Na,K-ATPase were not changed after IV-infection but protein levels significantly downregulated in the total cell lysate and the surface fraction of A/PR/8/1934(PR8) infected primary murine or human epithelial cells (AEC) as determined by Western blot and flow cytometry. Na,K-ATPase α1 surface expression was primarily decreased in non-infected cells or after treatment with conditioned media, and cocultivation of AEC with infected macrophages or recombinant TRAIL further triggered the decrease of Na,K-ATPase α1 surface expression levels. Na,K-ATPase downregulation depended on activation of protein kinase AMPK and its upstream kinase CaMKKβ and interference with this pathway after IV-infection by adenoviral delivery and overexpression of a dominant-negative form of AMPK could restore alveolar fluid clearance rates *in vivo*.
Additionally, Na,K-ATPase α1 was delocalized to the apical cell membrane in infected AEC and after transfection of the viral M2 segment, as shown by confocal microscopy. Na,K-ATPase colocalized and co-immunoprecipitated with the viral M2 protein.
We provide evidence that AFC in IV-infection is impaired by viral and host factors affecting Na,K-ATPase expression levels and localization. Targeting the underlying signalling pathways might provide targets for increasing edema clearance in IV-induced ARDS.

Keywords: Na,K-ATPase, alveolar fluid clearance, AMPK

The tetraspanin proteins, CD81 and CD9, promote efficient influenza A virusbudding through interactions with HA and NA

Yuhong Liang[1], Marie Lambelé[2], Markus Thali[2] and Megan L. Shaw[1]

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York, 10029
[2]Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, Vermont, 054053

Tetraspanins, broadly expressed in human cells, have been described to be involved at different stages of the life cycle for several viruses e.g. HIV-1, HCV. For influenza virus we have shown that CD9 and CD81 are incorporated into influenza virions. To further characterize the role of tetraspanins in the influenza viral cycle, we examined influenza A virus replication in cells with modulated level of tetraspanins. We find that CD9 and CD81, but not CD63, are required for optimal influenza A virus growth in tissue culture. We also examined influenza virus-like particles (VLPs) released from cells with modulated level of tetraspanins. We find that CD81 and CD9 (but not CD63) positively regulate VLP generation. With co-immunoprecipitation, we find that CD81 interacts with influenza A virus HA (H1, H2, H3 and H5) and NA (N1, N2). We also find that in the presence of CD81, CD9 interacts with influenza A HA and NA. We have mapped the interaction domain of NA with CD81 to specific residues and show that these mutants are unable to support VLP production. Also, we show that expression of a 50 residue portion of NA that interacts with CD81 has a dominant negative effect on VLP production, and that this can be rescued by CD9 overexpression. Overall our data suggest that certain tetraspanins play an important role during the influenza A virus life cycle, and particularly at the assembly/budding step where efficient trafficking of viral glycoproteins to the plasma membrane is critical for production of virus particles.

Keywords: Tetraspanin, virus budding, hemagglutinin, neuraminidase

Influenza Virus-induced Caspase-dependent Enlargement of Nuclear Pores Coincides with Nuclear Export of Viral Ribonucleoprotein Complexes

Dirk Mühlbauer[1†], Julia Dzieciolowski[1†], Martin Hardt[2], Andreas Hocke[3], Kristina L. Schierhorn[4], Susanne Herold[5], Thorsten Wolff[4], John Ziebuhr[1] and Stephan Pleschka[1*]

[1] Institute of Medical Virology, Justus Liebig University Giessen, Schubertstrasse 81, 35392 Giessen, Germany
[2] Imaging Unit, Biomedical Research Centre, Justus Liebig University Giessen, Schubertstrasse 81, 35392 Giessen, Germany
[3] Department of Internal Medicine/Infectious Diseases and Respiratory Medicine, Charité, Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany
[4] Division of Influenza and Other Respiratory Viruses, Robert Koch Institute, Seestrasse 10, 13353 Berlin, Germany
[5] Department of Internal Medicine (Pulmonology), University of Giessen and Marburg Lung Center, Klinikstrasse 36, 35392 Giessen, Germany

Influenza A virus (IAV) replicates its segmented RNA genome in the nucleus of infected cells and utilizes caspase-dependent nucleocytoplasmic export mechanisms to transport newly formed ribonucleoprotein complexes (RNPs) to the site of infectious virion production at the plasma membrane. Employing western blot and transmission electron microscopy (TEM) analysis we obtained evidence that apoptotic caspase activation in IAV-infected cells is linked to degradation of the nucleoporin Nup153, an integral subunit of the nuclear pore complex (NPC). Furthermore, TEM analysis of the NPC demonstrates a distinct enlargement of nuclear pores, thereby greatly extending the diffusion limit of nuclear pores and facilitating passive diffusion of larger protein complexes such as transient expressed GFP multimers. The study provides a possible explanation of the correlation between IAV-induced caspase activity and nuclear RNP export demonstrating how IAV exploits cellular structures and regulatory pathways, including intracellular transport mechanisms, to complete its replication cycle and maximize the production of infectious virus progeny.

Type I interferon signalling is inhibited upon influenza A virus and *Staphylococcus aureus* co-infection

K. Warnking[1], C. Klemm[1], B. Löffler[2], S. Niemann[2], G. Peters[2], S. Ludwig[1] and C. Ehrhardt[1]

[1]Institute of Molecular Virology (IMV), Center for Molecular Biology of Inflammation (ZMBE), Von Esmarch-Str. 56, 48149 Münster, Germany;
[2] Institute of Medical Microbiology, Domagkstr. 10, 48149 Münster, Germany

Influenza A viruses (IAV) are the causative agents of severe respiratory diseases, in some cases even leading to death. The majority of these fatal outcomes are linked to secondary bacterial pneumonia, caused by pathogens such as *Staphylococcus aureus* (*S. aureus*). While this problem is known for a long time, there is only scarce knowledge about the interplay of viruses with bacteria during infection on a molecular level. Thus, we assessed how *S. aureus* may interfere with IAV-induced signalling *in vitro*.
Co-infection of IAV and *S. aureus* results in enhanced type I interferon (IFN) expression in comparison to infection with the single pathogens alone. However, this increase in innate immune response does not lead to a reduction of viral titers, as expected. Instead we surprisingly observed higher viral titers in presence of *S. aureus*. Interestingly, IAV and *S. aureus* co-infection resulted in decreased mRNA levels of strictly IFN-dependent genes such as MxA or OAS as well as reduced ISG-15 protein expression. Based on these results we hypothesized that there might be a block of IFN-mediated signalling upon bacterial co-infection. In fact, we were able to detect an inhibitory effect of *S. aureus* infection on the type I IFN-mediated signalling on the level of STAT-1 phosphorylation, resulting in block of STAT1-STAT2 dimerization.
We provide deeper insights into the interplay of IAV and *S. aureus* regulating antiviral cellular strategies, resulting in enhanced progeny IAV.

Keywords: Influenza A viruses, S. aureus, co-infection

Note: Parts of these data were presented at different meetings before.

Session 3

INNATE IMMUNITY

Chair: Martin Schwemmle

Monday, September 22 2014

Keynote lecture: Innate immune sensing by RIG-I-like receptors and escape strategies of influenza virus
Michaela Gack, Boston, USA

DDX6 RNA Helicase is a novel RIG-I regulator that enhances RIG-I mediated IFN ß production
Rocio Davina Nunez, Berlin, Germany

Flu´s first defense against IFN: Viral suppressors of type I IFN response are prepackaged in influenza A virus virions
Swantje Liedmann, Münster, Germany

Comparison of the antiviral activity of M. spretus Mx1 and M. musculus A2G Mx1 against influenza A virus
Judith Verhelst, Gent, Belgium

DDX6 RNA Helicase is a novel RIG-I regulator that enhances RIG-I mediated IFN ß production

Rocio Daviña Nuñez, Matthias Budt, Sandra Sänger, Anne Sadewasser, Katharina Paki, Thorsten Wolff

Robert Koch-Institute, Division 17 Influenza Viruses and Other Respiratory Viruses, Berlin, Germany

Influenza virus infection is detected by the cellular pattern recognition receptor (PRR) retinoic acid inducible gene-I (RIG-I) receptor. (RIG-I)-like receptors recognize cytoplasmic viral RNA and induce the production of type I interferons (IFNs) and proinflammatory cytokines. To gain a better understanding of the role of RIG-I during influenza virus infection, we employed Stable-Isotopic-Labeling-of-amino-acids-in-Cell-Culture (SILAC), a mass spectrometric technique that allows relative protein quantification, to identify novel RIG-I interaction partners. Within the proteins that displayed an increased binding to RIG-I we found an enrichment of RNA binding proteins, among them, the RNA helicase DDX6.
DDX6 is an RNA helicase involved in mRNA metabolism and it is a component of P-bodies and stress granules. We confirmed the association between RIG-I and DDX6 by coimmunoprecipitation/immunoblot analysis of the endogenous proteins in infected and non-infected cells. Further we analyzed RIG-I and DDX6 distribution by confocal microscopy. DDX6 was localized in P-bodies, as expected, and presented an additional diffuse staining in the cytoplasm, that was similarly observed for RIG-I. In cells infected with an engineered influenza B virus DDX6 colocalized with RIG-I in stress granules. Interestingly transfection based reporter assays revealed that DDX6 overexpression enhances RIG-I mediate interferon ß promotor activation. These results were further confirmed by showing increased production of IFNß transcripts in infected cells upon DDX6 overexpression.
DDX6 is an RNA helicase involved in mRNA metabolism. Here we show that DDX6 is a novel RIG-I regulator that enhances its activation, suggesting a role for DDX6 in innate immunity.

Keywords: Influenza, RIG-I, DDX6, innate immunity, Mass spectrometry

Flu´s first defense against IFN: Viral suppressors of type I IFN response are prepackaged in influenza A virus virions

Swantje Liedmann[1], Eike R. Hrincius[2], Cliff Guy[3], Darisuren Anhlan[1], Rüdiger Dierkes[1], Gang Wu[4], Douglas R. Green[3], Thorsten Wolff[5], Jonathan A. McCullers[2,6], Stephan Ludwig[1] and Christina Ehrhardt[1]

[1]Institute of Molecular Virology (IMV), University of Muenster; [2]Department of Infectious Diseases, St. Jude Children's Research Hospital (SJCRH); [3]Department of Immunology, SJCRH; [4]Department of Computational Biology, SJCRH; [5] Division of Influenza and Other Respiratory Viruses, Robert Koch-Institut; [6]Department of Pediatrics, University of Tennessee Health Sciences Center.

The type I interferon (IFN) response represents the first line of defense to invading pathogens. Internalized viral ribonucleoproteins (vRNPs) of negative-strand RNA viruses provoke induction of an early IFN response by interacting with the cytosolic pathogen pattern receptor RIG-I and its recruitment to mitochondria.
We employed three-dimensional stochastic optical reconstruction microscopy (STORM) to visualize incoming influenza A virus (IAV) vRNPs as distinct helical structures associated with mitochondria. Co-localization with RIG-I and MAVS would suggest an early IFN response upon infection. However, no such response was detected. We observed that a distinct amino acid motif in the viral polymerases, PB1 and PA, suppresses early IFN induction. Mutation of this motif leads to reduced pathogenicity in vivo, while restoration increases it. Evolutionary dynamics in these sequences suggest that completion of the motif, combined with viral reassortment can contribute to pandemic risks.
In summary, we identified PB1 and PA as new players in the type I IFN inhibitory strategy evolved by IAV filling the gap in knowledge regarding early suppression of antiviral immune responses. In contrast to the NS1 protein, PB1 and PA are already present when internalized vRNPs are sensed by RIG-I and are located in close proximity to the site of RIG-I activation. Therefor, the suppression of the immediate anti-viral response is „prepackaged" in IAV in the sequences of vRNP-associated polymerase proteins.

Note: In parts presented at the 24th Annual Meeting of the Society for Virology, Alpbach, 2014.

Comparison of the antiviral activity of *M. spretus* Mx1 and *M. musculus* A2G Mx1 against influenza A virus.

Judith Verhelst[1,2], Jan Spitaels[1,2], Walter Fiers[1,2] and Xavier Saelens[1,2]

[1] VIB Inflammation Research Center, Technologiepark 927, Ghent, 9052 Belgium
[2] Department of Biomedical Molecular Biology, Ghent University, Technologiepark 927, Ghent, 9052 Belgium

The interferon-induced *Mx1* gene is an important part of the mammalian defense against influenza viruses. Mouse Mx1 inhibits influenza virus replication and transcription by suppressing viral polymerase activity present in ribonucleoproteins (RNPs). We showed previously that *Mus musculus* Mx1 interacts with two components of these RNPs, PB2 and nucleoprotein, and inhibits the interaction between these two proteins. To confirm these results and to identify regions in Mx1 that are important for inhibiting influenza infection, we compared the antiviral activity of *Mus musculus* A2G Mx1 with its ortholog in *Mus spretus*. Although *M. spretus* Mx1 is described to be at least as protective as A2G Mx1 *in vivo*, we found that its antiviral activity against PR8 was reduced *in vitro*. However, both Mx1 proteins showed a similar protection against an avian-type influenza virus. Sequence comparison exposed 25 amino acid differences, scattered over the entire Mx1 protein. Of these differences, two are present in loop L4, a region that is important for viral target recognition by human MxA. Replacing one of these residues in spretus Mx1 by the corresponding residue of A2G Mx1 increased the *in vitro* antiviral activity of spretus Mx1 against PR8. We also show that deletion of loop L4 abolished the antiviral activity of mouse Mx1. Taken together, these results indicate that loop L4 of mouse Mx1 is important for antiviral activity and viral target recognition, similar to its human homolog MxA. This suggests that Mx proteins from different mammals use a common mechanism to recognize influenza A viruses.

Keywords: Influenza, Mx1, Antiviral, Virus-Host interaction

Session 4

VIRAL REPLICATION STRATEGIES I – FROM ENTRY TO BUDDING

Chair: Klaus Schughart

Monday, September 22 2014

Mechanism of influenza virus uncoating
Yohei Yamauchi, Zürich, Switzerland

Single-molecule studies of promoter binding by the influenza RNA polymerase
Nicole Robb, Oxford, United Kingdom

Recruitment of RED-SMU1 complex by influenza A virus RNA polymerase to control viral mRNAsplicing
Guillaume Fournier, Paris, France

The nuclear export protein of H5N1 Influenza A Viruses recruits M1 to the viral ribonucleoprotein to mediate nuclear export
Linda Brunotte, Freiburg, Germany

Plasticity in the nucleoprotein of Influenza A virus to escape the antiviral Mx protein
David Riegger, Freiburg, Germany

Alterations of membrane curvature during influenza virus budding
Jeremy Rossman, Kent, United Kingdom

Mechanism of influenza virus uncoating

Yohei Yamauchi[1], Indranil Banerjee[1,2], Yasuyuki Miyake[3], Laure Decamps[1§], Hung Ho Xuan[1†], Peter Horvath[4], Amalio Telenti[5], Manfred Kopf[6], Patrick Matthias[3], Ulrike Kutay[1], Ari Helenius[1].

[1] Institute of Biochemistry, ETH Zürich, Otto-stern-Weg 3, CH-8093 Zürich, Switzerland
[2] Neurobiology, Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
[3] Epigenetics, Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
[4] Synthetic and Systems Biology Unit, Biological Research Center, H-6726 Szeged, Temesvári krt. 62, Hungary
[5] Institute of Microbiology, University Hospital Center and University of Lausanne, *CHUV* 1011 Lausanne, Switzerland
[6] Institute for Molecular Health Sciences, ETH Zürich, Otto-stern-Weg 7, CH-8093 Zürich, Switzerland
[§] Present address, Freiburg University, Germany
[†] Present address, University of Regensburg, Germany

Influenza A virus (IAV) is a serious human pathogen with great medical, social, and economic impact. The capsids of incoming IAVs must undergo uncoating before viral ribonucleoproteins (vRNPs) can be imported into the nucleus for replication. After hemagglutinin-mediated membrane fusion in late endocytic vacuoles, vRNPs and the matrix proteins, M1, dissociate from each other and disperse within the cytosol. Here we show that to disassemble its stable capsid shell for vRNP release, IAV takes advantage of the host cell's 1) aggresome formation and disassembly machinery, and 2) non-classical nuclear import pathway.

1) The virus mimics misfolded protein aggregates by carrying unanchored ubiquitin chains that serve as key signaling molecules to activate a histone deacetylase 6 (HDAC6)-dependent aggresome pathway. We observed that the free ubiquitins in the virus core interact with the ubiquitin-binding domain of HDAC6 and this was essential for uncoating and infection. A further requirement for the other components of the aggresome processing machinery such as dynein, dynactin, and myosin II, indicated that the physical force generated by microtubule (MT)- and actin-associated motors is crucial for IAV entry.
2) We found that M1 has a non-classical nuclear localization signal (NLS) sequence. This NLS is partly hidden at neutral pH in the M1-M1 dimer interphase. After exposure to acid inside endosomes, the full NLS sequence becomes accessible due to a conformational change in the dimer, enabling direct recognition by an importin. This results in the removal of M1 molecules from the surface of endosomes, promoting capsid disassembly. The vRNPs, on the other hand, employ classical NLS-mediated import, avoiding competition with M1.

Our current working model is that IAV uses mechanical forces provided by cytoskeletal motors and nucleocytoplasmic transport factors in order to uncoat its capsid. The clarification of such cell-assisted virus uncoating mechanisms can provide insights into new anti-viral strategies.

Single-molecule studies of promoter binding by the influenza RNA polymerase

Nicole C. Robb, Alexandra I. Tomescu, Narin Hengrung, Aartjan te Velthuis, Ervin Fodor and Achillefs N. Kapanidis

The genome of the influenza A virus comprises eight segments of single-stranded, negative-sense RNA with highly conserved 5' and 3' termini. These termini interact to form a double-stranded promoter structure that is recognized and bound by the viral RNA-dependent RNA polymerase (RNAP); however no 3D structural information for the influenza polymerase-bound promoter exists. Functional studies have led to the proposal of several 2D models for the secondary structure of the bound promoter, including a corkscrew model in which the 5' and 3' termini form short hairpins. We have taken advantage of an insect-cell system to prepare large amounts of active recombinant influenza virus RNAP, and used this to develop a highly sensitive single-molecule FRET assay to measure distances between fluorescent dyes located on the promoter and map its structure both with and without the polymerase bound. These advances enabled the direct analysis of the influenza promoter structure in complex with the viral RNAP, and provided 3D structural information that is in agreement with the corkscrew model for the influenza virus promoter RNA. Our data provide new insights into the mechanisms of promoter binding by the influenza RNAP and have implications for the understanding of the regulatory mechanisms involved in the transcription of viral genes and replication of the viral RNA genome.

Keywords: single-molecule, FRET, promoter, corkscrew, RNA polymerase

Recruitment of RED-SMU1 complex by influenza A virus RNA polymerase to control viral mRNAsplicing

Guillaume Fournier[1, 2, 3]*, Chiayn Chiang[1, 2, 3]*, Sandie Munier[1, 2, 3], Andru Tomoiu[1, 2, 3], Caroline Demeret[1, 2, 3], Pierre-Olivier Vidalain[2, 4], Yves Jacob[1, 2, 3], Nadia Naffakh[1, 2, 3]

[1] Institut Pasteur, Unité de Génétique Moléculaire des Virus à ARN, Département de Virologie, F-75015 Paris, France
[2] CNRS, UMR3569, F-75015 Paris, France
[3]Université Paris Diderot, Sorbonne Paris Cité, Unité de GénétiqueMoléculaire des Virus à ARN, EA302, F-75015 Paris, France
[4]Institut Pasteur, Unité de GénomiqueVirale et Vaccination, Département de Virologie, F-75015 Paris, France
* contributed equally to this work

Influenza A viruses are major pathogens in humans and in animals, whose genome consists of eight single-stranded negative RNA segments. Viral mRNAs are synthesized by the viral RNA-dependent RNA polymerase in the nucleus of infected cells, in close association with the cellular transcriptional machinery.Two proteins essential for viral multiplication, the exportin NS2/NEP and the ion channel protein M2, are produced by splicing of the NS1 and M1 mRNAs, respectively. Here we identify two human spliceosomal factors, RED and SMU1, that control the expression of NS2/NEP and are required for efficient viral multiplication. We demonstrate that in infected cells the hetero-trimeric viral polymerase recruits a complex formed by RED and SMU1, through direct interaction with its PB2 and PB1 subunits. We show that the splicing of the NS1 viral mRNA is specifically affected in cells depleted of RED or SMU1, leading to a decreased production of the spliced mRNA species NS2, and to a reduced NS2/NS1 protein ratio. In agreement with the exportin function of NS2, these defects impair the transport of newly synthesized viral ribonucleoproteins from the nucleus to the cytoplasm, and strongly reduce the production of infectious influenza virions. Overall, our results unravel a new mechanism of viral subversion of the cellular splicing machinery, by establishing that the human splicing factors RED and SMU1 act jointly as key regulators of influenza virus gene expression. In addition, our data point to a central role of the viral RNA polymerase in coupling transcription and alternative splicing of the viral mRNAs.

Keywords: Influenza virus, Alternative splicing regulation, RED-SMU1, RNA polymerase, virus-host interaction

The nuclear export protein of H5N1 Influenza A Viruses recruits M1 to the viral ribonucleoprotein to mediate nuclear export.

Linda Brunotte[1], Joe Flies[1], Hardin Bolte[1], Peter Reuther[1], Frank Vreede[2] and Martin Schwemmle[1]

[1] Institute of Virology, University Medical Centre Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany
[2] Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX 3RE, United Kingdom

Unlike most other RNA viruses, Influenza A Viruses replicate their genome in the nucleus of an infected cell. This requires nuclear entry and exit of incoming and newly synthesized viral ribonucleoproteins (vRNPs), respectively. Nuclear export of vRNPs is mediated by the viral matrix protein 1 (M1) and the nuclear export protein (NEP), which assemble on vRNPs in a daisy chain manner. In this complex M1 is described to recognize the newly synthesized vRNP by binding to NP and viral RNA. M1 is followed by NEP, which binds to vRNP-bound M1 with its C-terminus, while its NES-containing N-terminus mediates the interaction to the cellular nuclear export protein CRM1. Despite its nuclear export function NEP was recently discovered as a cofactor of the viral polymerase able to regulate its activity by binding to subunits PB1 and PB2. Interestingly, interaction of NEP with M1 and the viral polymerase are both mediated by its C-terminal moiety, raising the question if both functions could be associated. Here we can show that nuclear export of vRNPs requires the polymerase-enhancing function of NEP establishing a functional link between these two functions. In contrast to previous reports, we found that M1 alone is not able to interact with nuclear vRNPs but that this interaction depends on the presence of functionally active NEP. In conclusion, our data suggest a new refined model for the vRNP nuclear export complex in which the C-terminus of NEP simultaneously interacts with the viral polymerase and M1.

Plasticity in the nucleoprotein of Influenza A virus to escape the antiviral Mx protein

David Riegger[1], Mirco Schmolke[2,3], Rong Hai[2], Dominik Dornfeld[1], Martin Schwemmle[1], Adolfo García-Sastre[2,3,4], Georg Kochs[1]

[1]Institute of Virology, University Medical Center Freiburg, Germany.
[2]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, USA.
[3]Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, USA.
[4]Division of Infectious Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, USA.

The interferon-induced Mx-Protein is an important antiviral factor against influenza A viruses (FLUAV). Our previous work demonstrated that the viral nucleoprotein (NP) determines resistance of human seasonal and pandemic influenza viruses to Mx, while avian isolates are highly sensitive. We identified three amino acids (100I, 283P and 313Y) in a surface exposed area of the NP body domain of A/BM/1/1918 which are sufficient to gain Mx resistance.
For further investigations we generated a mouse-adapted A/PR/8/34 with changes of the Mx-resistant amino acids to the Mx-sensitive residues in NP (P283L and Y313F). This PR8(MxSensitive) virus was highly attenuated in Mx1-positive mice. To evaluate the capacity of PR8(MxSensitive) for escape from Mx restriction, we performed serial lung passages in Mx1-mice and identified an additional Y52N exchange in NP(P283L/Y313F). In vitro and in vivo analyses of these mutations showed, that the introduction of the 52N into PR8(MxSensitive) completely restores the Mx-resistance back to wildtype levels.
Intriguingly the NP of the recently emerged H7N9 that caused 150 deaths in China possesses an Asn-52 supporting reduced Mx sensitivity as we confirmed by cell culture and in vivo studies. Our data demonstrate that the surface exposed Mx-patch of NP is important for the virus to escape from the antiviral action of Mx and indicate a certain plasticity of NP in evading Mx restriction. Furthermore it suggests that Mx is an important barrier against zoonotic transmission and the overcome of this restriction by H7N9 partly contributes to its epidemic potential.

Keywords: H7N9, Mx-Protein, Influenza A, Nucleoprotein

Alterations of membrane curvature during influenza virus budding

A. Martyna and J. Rossman*

School of Biosciences, University of Kent, Canterbury, UK
*Presenting author

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 negative 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 additional viral proteins are capable of modifying membrane curvature and function to define and initiate the budding event whereas the M2 protein mediates membrane scission and completes the budding process through specific lipid and charge interactions.

Session 5

VACCINES & ANTIVIRALS

Chair: Stephan Ludwig

Tuesday, September 23 2014

Keynote lecture: Anti-inflammatory drugs for infectious diseases - a case for influenza
Mauro Teixeira, Belo Horizonte, Brazil

Sponsors talk: GP1681 as a Treatment for Influenza
Daryl Faulds (Gemmus Pharma Inc.), San Francisco, USA

Improvement of protection elicited by live attenuated influenza vaccines via mucosal TLR-3 ligation
Jose Vicente Perez-Giron, Hamburg, Germany

Sponsors talk: CiFlu, development of a novel subunit influenza vaccine candidate based on the ciliate performance expression system
Marcus Hartmann (Cilian AG), Münster, Germany

Novel small molecules inhibitors of influenza A and B viruses targeting the PA-PB1 subunits interaction of the viral polymerase
Arianna Loregian, Padova, Italy

GP1681 as a Treatment for Influenza

Daryl H. Faulds, Gemmus Pharma Inc. San Francisco, California, USA
Dale Barnard, Utah State University, Logan, Utah, USA
Ashley Dagley, Utah State University, Logan, Utah, USA
Bart Tarbet, Utah State University, Logan, Utah, USA
Hsiao-Lai Liu, Gemmus Pharma Inc. San Francisco, California, USA
Jiing-Huey Lin, Gemmus Pharma Inc. San Francisco, California, USA
William Guilford, Gemmus Pharma Inc. San Francisco, California, USA

Influenza A virus (IAV) infects the mammalian respiratory tract epithelium, which induces a vigorous inflammatory response from the immune system. The severity of the influenza infection is attributed to hypercytokinesis, therefore modulation of the host response and improved resolution of inflammation may be an effective treatment for IAV infections. At Gemmus we have identified the marketed drug, Beraprost (BPS), a modulator of the host immune response, as a possible treatment for influenza and other viral infections. Our lead candidate, GP1681, which is an isomer of BPS, has demonstrated cytokine modulating activity in human lymphocytes and the mouse influenza lethal challenge model.

In this presentation we describe results from in vitro studies which elucidate the signaling pathways involved in reduction of pro-inflammatory cytokines. We also describe the results from in vivo studies including a bacteria / virus co-infection model.

The isomer GP1681 has shown efficacy for treating influenza A H5N1 infections in mice, both stand-alone and in combination with low dose oseltamivir or an antibiotic. Because BPS is a repurposed drug and marketed for over 10 years in Asia for a different indication, BPS has been shown to be safe and well tolerated in man. The mechanism of action is known, and if marketed, GP1681 would be a new treatment for influenza virus infections.

Improvement of protection elicited by live attenuated influenza vaccines via mucosal TLR-3 ligation.

Pérez-Girón JV, [1]Belicha Villanueva A, Gómez-Medina S, Cruz JL, Hassan E, Lüdtke A, Ruibal P, [1, 2, 3,]García-Sastre A, Muñoz-Fontela C.

Heinrich Pette Institute, Leibniz Institute For Experimental Virology, Martinistrasse 52 20251 Hamburg, Germany. [1]Department of Microbiology, [2]Global Health and Emerging Pathogens Institute and [3]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1130, New York, New York 10029, USA.

Vaccine adjuvants are utilized to enhance the inflammatory response generated against a generally inert epitope that otherwise would be poorly immunogenic. However, their putative use to enhance or polarize the immune response to live-attenuated vaccines has not been explored. Mucosal administration of live-attenuated influenza vaccines (LAIVs) have the potential to generate CD8 T cells directed against broadly conserved influenza epitopes that can persist for the host lifetime. Because its ability to promote dendritic cell (DC) activation polyinosinic-polycytidylic acid (pI:C), a toll-like receptor (TLR)-3 ligand, has been extensively used as adjuvant of inactivated, dendritic-cell targeted, DNA, and subunit vaccines. Here we studied the effect of pI:C on CD8 T cell immunity and protection elicited by LAIVs. Mucosal treatment with poly IC shortly after vaccination enhanced rDC function, CD8 T cell formation, production of neutralizing antibodies as well as to reduce mortality and morbidity after PR8 lethal challenge. This adjuvant effect of pI:C was dependent on amplification of TLR3 signaling by non-hematopoietic radio-resistant stromal cells, and enhanced mouse protection to homosubtypic as well as heterosubtypic virus challenge. Our findings indicate that mucosal TLR3 ligation may be utilized to improve CD8 T cell responses to replicating vaccines, which has implications for protection in the absence of pre-existing antibody immunity.

CiFlu®: Development of a novel subunit influenza vaccine candidate based on the ciliate performance expression system.

Marcus Hartmann, Cilian AG, Münster

The critical annual manufacturing process for seasonal influenza vaccine based on embryonated chicken eggs, involves numerous steps and takes on average 6 to 8 months to complete. This often means that vaccine is only available late into the flu season. The timely availability of an effective influenza vaccine, at or before the flu season starts, is even more acute for vulnerable highest risk groups such as persons 65 years of age and older. The lack of timely availability of seasonal/pandemic vaccine has raised significant questions about the utility of the current, antiquated, cumbersome, expensive and unsafe manufacturing platform involving chicken eggs. Safety concerns about cell culture based virus proliferation processes called also alternative flu vaccines production processes into question.

Now new recombinant antigen manufacturing platforms were postulated to reduce production time and costs. Cilian's flu vaccine CiFlu® is a cost-effective subunit vaccine based on the heterologous expression of recombinant Influenza hemagglutinin (rHA) in the ciliate *Tetrahymena*. Utilizing its CIPEX-System as such a manufacturing platform, Cilian has successfully demonstrated repeated expression of rHA at high yield: four subunit vaccines has been expressed and shown to be functionally active. Mice were first immunized with the monovalent rHA. HA antibodies were harvested and its ability to inhibit the respective influenza strain was tested. The results demonstrated comparable or better efficacy (*in vivo* inhibitory immunogenicity) to monovalent vaccine from chicken eggs. Cilian meanwhile received a positive scientific advice from the German Paul Ehrlich Institute for CiFlu® and is developing a comprehensive clinical plan.

Keywords: influenza subunit vaccine, recombinant production, ciliate, Tetrahymena

Novel small molecules inhibitors of influenza A and B viruses targeting the PA-PB1 subunits interaction of the viral polymerase.

Giulio Nannetti[1], Giulia Muratore[1], Beatrice Mercorelli[1], Laura Goracci[2], Serena Massari[3], Susan Lepri[2], Paul Digard[4], Gabriele Cruciani[2], Oriana Tabarrini[3], Giorgio Palù[1], and Arianna Loregian[1]

[1]Department of Molecular Medicine, University of Padua, Padua, Italy; [2]Department of Chemistry, University of Perugia, Perugia, Italy; [3]Department of Chemistry and Technology of Drugs, University of Perugia, Perugia, Italy; and [4]Roslin Institute, University of Edinburgh, Easter Bush, Midlothian, United Kingdom.

Influenza viruses are responsible for yearly epidemics and pandemics characterized by high morbidity and significant mortality. The incomplete protection provided by vaccines and the rapid emergence of resistance against the current antivirals highlights the need for the development of novel drugs. Recently, we identified new anti-influenza compounds from an *in silico* screening, able to specifically interfere with the protein-protein interactions between the PA and PB1 subunits of influenza A virus RNA polymerase and also to inhibit influenza virus replication (Muratore *et al.*, PNAS 2012, 109:6247-52). Among these compounds, some hits were selected for the design and synthesis of analogs, in order to increase the potency of the compounds. Some of the analogues were non-cytotoxic and more potent than the original compounds (Massari, Nannetti *et al.*, J. Med. Chem. 2013; Lepri, Nannetti *et al.*, J. Med. Chem. 2014). In addition, these analogues effectively inhibited the replication of a panel of influenza A and B virus strains, including an oseltamivir-resistant isolate, thus demonstrating to possess broad-spectrum anti-influenza activity. Finally, for the best derivates, we made predictions and simulations of their interaction with the target. This has allowed us to delineate the structure-activity relationships and to generate a pharmacophoric model that could be useful for further optimization of these inhibitors (Lepri, Nannetti *et al.*, J. Med. Chem. 2014). Importantly, preliminary studies indicate that these small molecules also exhibit considerably lower propensity to develop drug resistance than other anti-influenza compounds, e.g. oseltamivir and rimantadine. Taken together, our results provide the basis for the development of a new generation of anti-influenza agents.

Keywords: antivirals, RNA polymerase, disruption of protein-protein-interaction

Session 6

VIRAL REPLICATION STRATEGIES II - THE VIRAL NS1 PROTEIN

Chair: Stephan Pleschka

Tuesday, September 23 2014

Influenza A/NS1 mediated inhibition of the signaling integrator c-Abl results in excessive lung homeostasis deregulation, lung damage and mortality
Eike R. Hrincius, Memphis, USA

Avian influenza NS1 protein inhibits innate responses in vitro, but is unable to suppress an in vivo cytokine storm
Konrad Bradley, London, United Kingdom

Quantitative proteomic analysis of the Influenza A virus protein NS1 during natural cell infection identifies PACT as an NS1 target protein and antiviral host factor
Hans Haecker, Memphis, USA

Influenza A/NS1 mediated inhibition of the signaling integrator c-Abl results in excessive lung homeostasis deregulation, lung damage and mortality.

Eike R. Hrincius[1], Swantje Liedmann[2], Peter Vogel[3], David Finkelstein[4], Christina Ehrhardt[2], Stephan Ludwig[2], Jonathan A. McCullers[1,5]

[1]Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN USA
[2]Institute of Molecular Virology (IMV), University of Muenster, Muenster, Germany
[3]Department of Veterinary Pathology, St. Jude Children's Research Hospital, Memphis, TN USA
[4]Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN USA
[5]Department of Pediatrics, University of Tennessee Health Sciences Center, Memphis, TN, USA

The non-structural protein 1 (NS1) of influenza A viruses (IAV) modulates immune signaling. Our earlier studies have shown that NS1 proteins from avian IAV are capable of inhibiting the key signaling integrator c-Abl, resulting in massive cytopathic cell alterations. In the current study, we addressed the consequences of NS1-mediated alteration of c-Abl in an in vivo model. Comparing two isogenic influenza A viruses only differing in their ability to inhibit c-Abl, we observed clearly elevated morbidity and mortality for the c-Abl inhibiting virus. Interestingly, virus growth and host immune responses were comparable, demonstrating that the mechanism(s) of increased pathogenicity are replication and immune response independent. Analysis of lung damage demonstrated that the NS1-mediated block of c-Abl resulted in severe lung pathology and massive edema formation. Marked bronchiolar epithelium denudation and loss of type II pneumocytes were observed. In line with the histology findings, microarray analysis revealed extensive deregulation of genes involved in cell integrity and vascular endothelial regulation. In conclusion, NS1 protein blockade of c-Abl signaling drives acute lung injury in a replication and immune response independent fashion. Several candidate pathways for further exploration of these novel effects have been identified and the concept of disturbed lung homeostasis, acute lung injury and systemic effects like reduced oxygen supply and kidney failure will be broadly investigate in viral and bacterial infections. The presented data illustrate an entirely new mechanism of NS1 mediated pathology distinct from its canonical functions.

This work was supported by ALSAC and DFG.

Avian influenza NS1 protein inhibits innate responses *in vitro*, but is unable to suppress an *in vivo* cytokine storm.

Konrad C Bradley, Lorian CS Hartgroves, Aisling Vaughan, Rebecca Cocking, Jonathan W Ashcroft, Ruth Elderfield, Holly Shelton, and Wendy S Barclay

Section of Virology, Division of Infectious Disease, Imperial College London, St. Mary's Campus, Norfolk Place, London, United Kingdom

The requirements for avian-human transmission of highly pathogenic avian influenza viruses is a source of intense research, in part due to the 60% case fatality rate associated with these strains. The significant morbidity and mortality caused by avian strains is typically due to an exacerbated immune response. This is commonly referred to as a "cytokine storm," which occurs despite the presence of NS1, the main interferon antagonist protein encoded by influenza. We sought to understand the role of the internal proteins encoded by influenza in the induction of a cytokine storm, particularly to ask whether deficits in the immunomodulatory capacity of the avian NS1 might explain the high cytokine levels induced by these viruses. We found that, in comparison with NS1 protein from pandemic 2009 H1N1 virus, NS1 protein of the clade 2 avian influenza virus A/Turkey/Turkey/05/2005 was able to efficiently suppress interferon induction in A549 lung epithelial cells both when expressed exogenously or in the context of infection virus. Interestingly, the same H5 NS1 protein did not suppress the cytokine storm induced by viral infection of mice when paired with the homologous H5N1 polymerase set, whereas mice infected with a recombinant virus pairing the H5 NS gene segment with the polymerase genes from pH1N1 2009 virus did not display hyperinduction of cytokines. These results suggest avian influenza NS1 proteins may be able to inhibit an early antiviral response, but one or more of the other internal genes from H5N1 avian virus are responsible for the lethal runaway immune response.

Keywords: H5N1, NS1, cytokine storm

Quantitative proteomic analysis of the Influenza A virus protein NS1 during natural cell infection identifies PACT as an NS1 target protein and antiviral host factor

Kazuki Tawaratsumida, Van Phan, Eike R. Hrincius, Anthony High, Richard Webby, Vanessa Redecke and Hans Häcker

St. Jude Children's Research Hospital

Influenza A virus (IAV) replication depends on interaction of virus proteins with partially characterized host factors, and the non-structural protein 1(NS1) is essential in this process by targeting diverse cellular functions including the type I interferon (IFN-I) response. In order to identify host proteins targeted by NS1, we established a replication-competent recombinant IAV that expresses epitope-tagged forms of NS1 and NS2, which are encoded by the same gene segment, allowing purification of NS proteins during natural cell infection and analysis of interacting proteins by quantitative mass spectrometry (MS). We identified known NS1- and NS2-interacting proteins, but also uncharacterized proteins, including PACT, an important co-factor for the IFN-I response triggered by the viral RNA-sensor RIG-I. More detailed analysis shows that NS1 binds PACT during virus replication, preventing efficient PACT/ RIG-I interaction and IFN-I activation. While a mutant virus with deletion of NS1 induced high levels of IFN-I in control cells, as expected, shRNA-mediated knock-down of PACT compromised IFN-I activation. These observations are consistent with the interpretation that PACT is essential for IAV recognition, which is compromised by NS1. Together, our data describe a novel approach to identify virus-host protein interactions and demonstrate that NS1 interferes with PACT, whose function is critical for robust IFN-I production.

Keywords: non-structural proteins, proteomics, interferon, RIG-I, PACT

Session 7

PATHOGENESIS

Chair: Roland Zell

Tuesday, September 23 2014

Keynote lecture: Novel Influenza A (H7N9) Virus: Origin "Host Jump" and Drug-Resistance
George G. Gao, Beijing, China

H5N1 and H7N9 Viruses Induce Impaired IFN-mediated Antiviral State by Different Mechanisms
Pamela Österlund, Helsinki, Finland

The PA-X protein modulates virus pathogenicity in chick embryos independently of host cell shut off function
Saira Hussain, Edinburgh, Scotland

Activation of Influenza Virus Hemagglutinin by a *L. pneumophila* Protease
Annika Arendt, Marburg, Germany

H5N1 and H7N9 Viruses Induce Impaired IFN-mediated Antiviral State by Different Mechanisms

Veera Arilahti[(1)], Sanna M. Mäkelä[(1)], Janne Tynell[(1)], Ilkka Julkunen[(1,2)] and Pamela Österlund[(1)]

[(1)]National Institute for Health and Welfare, Virology Unit, Helsinki, Finland
[(2)]University of Turku, Department of Virology, Turku, Finland

The emergence of human infections with avian influenza A(H7N9) virus in China in 2013 has evoked the concern of avian influenza as the source of the next pandemic virus. H7N9 virus has now caused two epidemic clusters following the seasonal pattern seen with human influenza. Most H7N9 virus-infected patients suffer from acute respiratory distress syndrome (ARDS) and severe lower airway inflammation similar to that seen in patients with H5N1 virus infection. This raises a question whether H7N9 virus can induce a similar immune dysregulation as H5N1 virus, which contributes to the devastating fatal outcome of the disease. We compared the antiviral-inducing mechanisms during the infection with seasonal H3N2 and avian-origin H5N1 and H7N9 influenza viruses in human immune cells. We observed that the novel H7N9 virus, although replicating strongly, did not efficiently induce antiviral IFN or proinflammatory cytokine responses, and this deficiency was associated with block in IRF3 activation. H5N1 virus, instead, was inducing dysregulation of the IFN and cytokine genes presumably by activating multiple transcription factor systems, such as IRFs and NFκB, through incoming viral material. Interestingly, in H5N1 infection, in spite of the massive IFN induction the expression levels of MxA and other antiviral proteins remained relatively low. This suggests that the H5N1 virus is able to reduce the antiviral state by blocking the expression of IFN-stimulated genes (ISGs). Our data suggests that although both H7N9 and H5N1 avian-origin viruses cause severe pneumonia and ARDS in humans, the underlying mechanisms behind the pathology are distinct.

Keywords: H5N1, H7N9, avian influenza, human host responses, interferon

The PA-X protein modulates virus pathogenicity in chick embryos independently of host cell shut off function.

Saira Hussain[1], Helen Wise[1, 2], Anne Lo[2], G. Louise Bell[2], Brett Jagger[2,3], Pip Beard[1], Andrew Firth[2], Jeffery K. Taubenberger[3], Othmar Engelhardt[4], Paul Digard[1, 2]

[1]The Roslin Institute, University of Edinburgh, EH25 9RG, UK; [2]The Division of Virology, University of Cambridge, CB2 1QP, UK; [3]Viral Pathogenesis and Evolution Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA; [4]Division of Virology, National Institute for Biological Standards and Control, EN6 3QG, UK.

The recently identified PA-X protein of influenza A virus has proposed roles in host cell shut off and viral pathogenesis. Although most strains of virus are predicted to encode PA-X, strain-dependent variations in activity have been noted, with laboratory-adapted isolates lacking effective shut off function. We found that while in vitro expression of the laboratory adapted A/PR/8/34 (PR8) PA-X protein was comparable to that of an avian virus A/chicken/Rostock/34 (FPV) PA-X, the PR8 protein had much lower repressive function, in both mammalian and avian cells. Loss of normal PA-X expression, either by mutation of the frameshift site or by truncating the X-ORF, had little effect on the infectious titre of virus grown in mammalian cells or embryonated hens eggs. However, mutation of PA-X led to decreased embryo mortality and lower overall gross pathology; effects that were more pronounced in the WT PR8 strain than in a 7+1 reassortant with the FPV PA segment, despite the FPV PA-X protein having a more potent shut off activity. This suggests that the effects of PA-X on virus pathogenicity are host dependent and that the PR8 PA-X protein may harbour a function unrelated to host cell shut off.

Activation of Influenza Virus Hemagglutinin by a *L. pneumophila* Protease

Annika Arendt, Wolfgang Garten, Hans-Dieter Klenk and Eva Böttcher-Friebertshäuser

Institute of Virology, Philipps-University Marburg, Marburg, Germany

Bacterial coinfections are one of the major causes for complications of influenza and contribute significantly to morbidity and mortality. Viral infection has been shown to prime the respiratory epithelium for attachment and colonisation of bacteria such as *S. pneumonia* and *S. aureus*. Interestingly, certain bacteria have been demonstrated to support proteolytic activation of influenza virus directly by secreting a protease that is able to cleave the hemagglutinin (HA) precursor HA_0 in its fusion competent form (HA_1 and HA_2). Thereby bacteria may contribute to virus spread and enhanced viral pathogenicity.
Here, we were able to show that *Legionella pneumophila* secretes a protease in liquid culture that is able to cleave the HA of influenza virus in *vitro*. Interestingly, different cleavage patterns were observed for distinct virus subtypes. Some subtypes of HA were cleaved similar to trypsin, that was used as a positive control. Furthermore the infectivity of these viruses was increased after treatment with *L. pneumophila* supernatant. For other subtypes, atypical cleavage fragments were observed, and infectivity was not enhanced. Taken together we could show that *Legionella pneumophila* secretes a protease which is able to cleave and functionally activate the influenza virus HA of specific subtypes. Identification and further characterization of the protease is currently ongoing.

Keywords: influenza virus hemagglutinin; proteolytic cleavage; viral-bacterial coinfection

POSTER PRESENTATIONS

P1

An infectious bat chimeric influenza virus harboring the entry machinery of a influenza A virus

Mindaugas Juozapaitis (1), Étori Aguiar Moreira (1,2,3), Ignacio Mena (4,5), Sebastian Giese (1,3), David Riegger (1), Anne Pohlmann (6), Dirk Höper (6), Gert Zimmer (7), Martin Beer (6), Adolfo García-Sastre (4,5,8) and Martin Schwemmle (1)

1 Institute for Virology, University Medical Center Freiburg, Germany.
2 Speeman Graduate School of Biology and Medicine, University of Freiburg, Germany.
3 Faculty of Biology, University of Freiburg, Germany.
4 Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, USA
5 Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, USA
6 Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald - Insel Riems, Germany.
7 Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland
8Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, USA

In 2012 the complete genomic sequence of a new and potentially harmful influenza A-like virus from bats (H17N10) was identified. However, infectious influenza virus was neither isolated from infected bats nor reconstituted, impeding further characterization of this virus. Here we show the generation of an infectious chimeric virus containing six out of the eight bat virus genes, with the remaining two genes encoding the HA and NA proteins of a prototypic influenza A virus. This engineered virus replicates well in a broad range of mammalian cell cultures, human primary airway epithelial cells and mice, but poorly in avian cells and chicken embryos without further adaptation. Importantly, the bat chimeric virus is unable to reassort with other influenza A viruses. Although our data do not exclude the possibility of zoonotic transmission of bat influenza viruses into the human population, they indicate that multiple barriers exist that makes this an unlikely event.

P2

Full virulence of avian influenza H7N1 virus in chickens requires a combination of multibasic proteolytic cleavage site and mutations in the HA2 protein

El-Sayed M. Abdelwhab, Jutta Veits, Kerstin Tauscher, Jens Teifke, Jürgen Stech and Thomas C. Mettenleiter

The Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Insel-Riems, Greifswald, Germany

Outbreaks of low pathogenic avian influenza (LPAI) H7N1 virus in poultry in Italy in March 1999 were marked as the largest epidemic of LPAI in Europe. In December, 1999 a highly pathogenic avian influenza (HPAI) H7N1 virus emerged causing severe economic losses in the poultry industry. Sequence analysis of the LPAI and HPAI viruses revealed mutations in all gene segments where the hemagglutinin (HA) of the HPAI virus has a peculiar multibasic proteolytic cleavage site (mCS) among all influenza subtypes. Yet, the genetic basis of virulence of this virus remains almost entirely unknown. In this study, LPAI and HPAI H7N1 viruses were cloned using reverse genetics and mutants possessing point mutations in the HA of LPAI and HPAI viruses were used for experimental infection of chickens as well as replication kinetics in cell culture. The results indicated that HPAI H7N1 viruses with monobasic CS or the whole HA of LPAI H7N1 virus were avirulent for chickens resembling the LPAI virus. On the contrary, LPAI virus containing the whole HA of HPAI virus was as virulent and transmissible as the HPAI strain where all infected and contact chickens died. Meanwhile, a recombinant LPAI virus carrying mCS was remarkably less virulent than the HPAI virus. Mutations in the HA2, mostly adjacent to the cleavage site, were required for the exhibition of full virulence in chickens although it did not influence tissue tropism and amount of virus excretion in infected birds or the replication of the virus in cell culture.

Keywords: Avian influenza virus, H7N1, cleavage site, virulence, transmissibility, chickens

P3

The influence of previous infection or vaccination prior to the intranasal vaccination against influenza virus infection

Kazuyuki Ikeda[1], Ryo Ito[1], Akira Ainai[2, 1], Tadaki Suzuki[1], Yuki Ohara[1], Shinji Saito[1], Shin-ichi Tamura[1], Takato Odagiri[2], Masato Tashiro[2], Hideki Asanuma[2, 1], Hideki Hasegawa[1, 2]

[1] Department of Pathology, and [2] Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Intranasal vaccination against influenza virus induces both IgG antibodies in serum and secretory IgA antibodies in the nasal mucosa. These IgA antibodies are superior to IgG Antibodies in serum with respect to protection against infection. However, this has mainly been demonstrated in mice, those are naïve to influenza virus, whereas human adults have immunological memory induced by previous infection or vaccination. In this study, the influence of previous infection or vaccination upon the antibody responses induced by the following vaccination was investigated in mice.
BALB/c mice were infected or vaccinated with A/Narita/1/09 (A/NRT, A(H1N1)pdm09) or A/Puerto Rico/8/34 (A/PR8, A(H1N1)) strain, 3 weeks before the following vaccination with A/NRT. Vaccination was performed by intranasal or subcutaneous administration with an inactivated vaccine. Two weeks after the last vaccination, all mice were challenged with A/NRT. At 3 days post-infection, serum and nasal wash specimens were collected to determine antibody levels and virus titers.
A previous infection, rather than vaccination, with A/NRT or A/PR8 tended to induce a high amount of antibodies after the following vaccination. However, intranasal vaccination with A/NRT was superior to subcutaneous vaccination in both the reduction of virus titers and the induction of A/NRT hemagglutinin-specific IgA antibodies in nasal washes among mice previously exposed to A/NRT or A/PR8. Thus, even if mice have previously been infected or vaccinated, the following intranasal vaccination is considered to be superior to subcutaneous vaccination in the induction of protective immunity. At present, the difference of nasal neutralizing antibody response is continuously investigated.

Keywords: intranasal vaccination, A(H1N1)pdm09, secretory IgA antibody

P4

Glycosylation but not Receptor-Binding Contributes to Pathogenicity of and Immune Responses to H3N2 Influenza A Viruses

Irina V. Alymova,[1, 2] Ian A. York,[1] Shane Gansebom,[2] and Jonathan A. McCullers[2,3]

[1]Influenza Division, National Center for Immunization & Respiratory Diseases, Centers for Disease Control & Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA; [2]Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA;
[3]Department of Pediatrics, University of Tennessee Health Sciences Center, 50 N Dunlap St, Memphis, TN 38103, USA

Since its emergence as a pandemic virus in 1968, influenza A H3N2 has become established as a seasonal epidemic strain. The molecular evolution of this virus has included the progressive addition of *N*-linked glycans onto the hemagglutinin globular head. Previously, we demonstrated differences in pathogenicity and induction of immune response in mice between the H3N2 pandemic virus A/Hong Kong/1/1968 (HK68) and variants with either two (residues 63 and 126: HK68+2) or four (residues 63, 126, 133, and 246: HK68+4) additional glycosylation sites on the hemagglutinin globular head. Here, we evaluated the consequences of this incremental glycosylation on sialic acid (SA) receptor-binding. Wild-type HK68 bound to a selection of both α2-3- and α2-6-linked SAs on a glycan array. In contrast, HK68+2 revealed a more restricted binding profile. It bound to 80% fewer glycans of both linkages than wild-type HK68 resulting in 90% overall signal loss for whole profile. Interestingly, HK68+4 binding to SAs was near that of the wild-type virus. Variations in viruses' receptor-bindings as measured by glycan array were confirmed by their elution patterns from erythrocytes. Despite high level of binding, infection of mice with HK68+4 resulted in marked decreases in morbidity, virus lung titers, and antibody responses, while HK68+2 (showing lowest level of binding) was more similar to wild-type HK68 in these properties. We conclude that the level of glycosylation on the hemagglutinin globular head can alter the pathogenicity and immunogenicity of influenza A H3N2 viruses, but that these differences are not related to SA receptor binding properties.

Keywords: influenza/hemagglutinin/receptor-binding/glycan/array

P5

Relationship between innate immune responses and pathogenicity of influenza H7N9 virus that is adapted to mice

Hideki Asanuma[1)2)], Akira Ainai[1)2)], Noriyo Nagata[2)], Masato Tashiro[1)], Takato Odagiri[1)]

[1)] Influenza Virus Research Center, National Institute of Infectious Diseases
[2)] Department of Pathology, National Institute of Infectious Diseases

A new type of avian influenza A (H7N9) virus was reported in China in 2013. We demonstrated that A/Anhui/1/2013 (A/Anhui; H7N9) virus could be adapted to grow in mice and that the lethality of mouse-adapted A/Anhui was about 1000 times higher than that of the original strain. Next, after virus inoculation in lungs of mice, innate immune responses were investigated by quantitative PCR array of type I IFN related genes.
In the case of infection with a non-lethal dose of mouse-adapted A/Anhui, the expression of type I IFN related genes in the lungs was similar to that in mice given original A/Anhui. In both cases almost no changes in type I IFN related genes were found 1 day after infection and a remarkably high expression of the related genes was shown 3 days after the infection. Interestingly, although in mice given a lethal dose of original A/Anhui virus, this high expression of type I IFN related genes was already detected 1 day after infection, in mice given a lethal dose of mouse-adapted A/Anhui, such a high expression of these genes failed to be shown. These results suggest that adaptation of A/Anhui to mice not only results in a strong increase in pathogenicity but also leads to an alternative activation of the innate immune responses. Currently, we are investigating which key factor is involved in the induction of increased pathogenicity after adaptation in mice.

Keywords: Influenza H7N9 virus, mice adaptations, innate immune responses, cytokine, pathogenicity

P6

Evidence of a New Mechanism of Interferon Induction in IAV Infection Triggered by a Defective Interfering RNA-encoded Protein

Boergeling, Y.[1]; Rozhdestvensky, T.[2]; Schmolke, M.[1,4]; Robeck, T.[2]; Randau, G.[2]; Wolff, T.[3]; Brosius, J.[2]; and S. Ludwig[1]

[1]Institute of Molecular Virology (IMV), [2]Institute of Experimental Pathology, [1,2]Center for Molecular Biology of Inflammation (ZMBE), University of Muenster, Germany; [3]Robert Koch Institute, Berlin, Germany; [4]present address: Department of Microbiology and Molecular Medicine, Medical Faculty, University of Geneva, Switzerland

Influenza A virus (IAV) defective interfering (DI) RNAs are generated as a byproduct of the error-prone viral RNA replication. They are commonly derived from the larger segments of the viral genome and harbor deletions of different sizes resulting in the generation of replication incompatible viral particles. Furthermore, DI RNAs are known to be strong inducers of RIG-I-dependent type I interferon (IFN) responses. The present study identifies a novel DI RNA derived from the PB2 segment of the H5N1 strain A/Thailand/1(KAN-1)/2004. It encodes a 10 kDa protein ($PB2_{\Delta}$) sharing the N-terminal amino acid sequence of the parental PB2 protein. $PB2_{\Delta}$ induces the expression of IFNβ and IFN-stimulated genes by direct interaction with the cellular adapter protein MAVS, thereby reducing viral replication of IFN-sensitive viruses such as influenza or vesicular-stomatitis virus. This induction of IFN is completely independent of DI RNA serving as pathogen-associated pattern and thus does not require the cytoplasmic sensor RIG-I. These data suggest that not only DI RNAs but also DI RNA-encoded proteins can act immunestimulatory and might therefore contribute to the overwhelming cytokine response characteristic for highly pathogenic H5N1 viruses. This is the first time that such a function was described for a DI RNA-encoded protein, a finding that has several important implications with regard to deciphering viral protein functions and options for immunestimulatory approaches. Furthermore, this is an example of how influenza viruses may acquire novel polypeptides with altered functions from its limited genome.

Keywords: defective interfering (DI) RNA, DI RNA-encoded protein, type I interferon, MAVS

P7

Expression of adenoviral early-region 1A protein benefits cell culture-based influenza vaccine production

Mandy Bachmann[1*], Timo Frensing[1,2], Volker Sandig[3], Udo Reichl[1,2]

[1] Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Magdeburg, Germany
[2] Otto von Guericke University, Chair of Bioprocess Engineering, Magdeburg, Germany
[3] ProBioGen AG, Berlin, Germany
*Correspondence: bachmann@mpi-magdeburg.mpg.de

Influenza viruses are a major threat of human health and cause worldwide 3 to 5 million cases of severe illness every year. To prevent morbidity and mortality caused by influenza virus epidemics and pandemics, an annual vaccination is required. Since the global demand for influenza vaccines is continuously increasing, mammalian cell cultures are an attractive alternative to egg-based production systems due to better flexibility and scalability. Different human-derived production cell lines such as PER.C6 (Crucell), HEK293SF and AGE1.HN (ProBioGen AG) are currently tested as substrate for the cultivation of influenza viruses. Unfortunately, cellular defense mechanisms and apoptosis induction interfere with virus replication and shorten the life span of virus producing cells.
To study the impact of the antiviral response on virus yields using human production cell lines, we stimulated interferon signalling in A549 (adherent), HEK293 (adherent), HEK293SF (suspension) and AGE1.HN (suspension) cells. Our results showed an impaired antiviral response in HEK293, HEK293SF and AGE1.HN cells compared to A549 cells. Subsequent experiments demonstrated that the expression of adenoviral early-region 1A protein in Ad5-transformed cell lines suppressed interferon signaling. These results indicate a potential advantage of Ad5-transformed cell lines for influenza vaccine production.

Keywords: influenza virus, immune response, vaccine production, cell culture

P8

Evaluation of serine proteases and serine protease inhibitors as human biomarkers to distinguish between influenza A virus and *Streptococcus pneumonia*e infections

Mahmoud M. Bahgat[1&2] and Frank Pessler[1]

[1]Research Group Biomarkers for Infection and Immunity, Institute of Experimental Infection Research, TWINCORE Centre for Experimental and Clinical Infection Research, Hannover, Germany [2]Research Group Immunology and Infectious Diseases, the National Research Centre, Cairo, Egypt

The known acute phase proteins to be elevated during pneumonia cannot appropriately distinguish between bacterial and viral etiology of the disease. For example C-reactive protein is highly sensitive but non-specific. Although procalcitonin is *S. pneumoniae* specific it can only detect systemic infections, and sensitivities of the commercially available detection assays vary widely. Therefore, there is an urgent need to identify new human biomarkers which can distinguish between bacterial and viral pneumonia. Serine proteases are known to mediate the initial phase of influenza infection. Also, members of the same enzyme family have been implicated in end-organ damage in severe systemic *S. pneumoniae* infections. Therefore, we studied the expression profile of tans-membrane serine proteases (TMPRSS) and serine protease inhibitors (SERPINS) at the protein level in peripheral blood mononuclear cells (PBMCs) from healthy humans upon infection with PR8-H1N1 influenza virus or *S. pneumoniae,* or upon coinfection with both pathogens, in comparison to healthy uninfected cells. Zymography results clearly showed distinct protease profiles in culture media of PBMCs in response to H1N1 virus and *S. pneumoniae* infections. TMPRSS 2, 3 and 5 were significantly elevated in response to H1N1 virus infection while TMPRSS4 was significantly elevated in response to co-infection. SERPIN3 was down regulated early after *S. pneumoniae* infection and was significantly elevated at 24 h post bacterial infections. These results suggest the potential of TMPRSS and SERPINS as potential biomarkers to distinguish between viral and bacterial infection and encourage further testing of expression levels of members belonging to both protein families in clinical samples from patients with community acquired pneumonia.

P9

Use of deep-sequencing to evaluate the intrinsic heterogeneity of human influenza A viruses directly in nasal swabs

C. Barbezange[1,2], H. Blanc[1], O. Isakov[4], V. Enouf[2,3], N. Shomron[4], S. van der Werf[2,3], M. Vignuzzi[1]

[1]Institut Pasteur, Viral Populations and Pathogenesis, CNRS UMR 3569, Paris, France; [2]Institut Pasteur, Molecular Genetics of RNA Viruses, CNRS UMR 3569, University Paris Diderot, Paris, France; [3]Institut Pasteur, National Influenza Centre, Paris, France; [4]University of Tel-Aviv, Sackler Faculty of Medicine, Department of Cell and Developmental Biology, Tel-Aviv, Israel

After a pandemic wave in 2009 following its introduction in the human population, H1N1pdm09 subtype replaced the previously circulating H1N1 virus and, along with the H3N2 subtype, is now responsible for the seasonal type A influenza epidemics. So far, the evolutionary potential of influenza viruses has been mainly documented by consensus sequencing data. However, RNA virus polymerases are considered to have low fidelity, and a virus thus exists as a cloud of closely related sequences, which could influence its fitness and its adaptability. Interest in this quasispecies nature has increased with the development of next-generation sequencing (NGS) technologies that allow a wider study of the genetic variability. NGS deep-sequencing methodologies were developed to determine the whole-genome genetic heterogeneity of the 3 subtypes of influenza type A viruses that circulated in humans between 2007 and 2012 in France, directly from nasal swab samples. We are presenting here the results of the comparison performed to identify subtype/severity signatures focusing on mutation frequencies and specific nucleotide polymorphisms.

Keywords: NGS, diversity, seasonal, quasispecies

P10

Chimeric NP non coding regions between type A and C influenza viruses reveal their role in translation regulation.

B. Crescenzo-Chaigne[1], C. Barbezange[1], V. Frigard[1], D. Poulain[1] and S. van der Werf[1,2]

[1]Institut Pasteur, Molecular Genetics of RNA Viruses, CNRS UMR 3569, University Paris Diderot, Paris, France; [2]Institut Pasteur, National Influenza Centre, Paris, France

For each genomic viral RNA (vRNA) composing the segmented genome of influenza viruses, the coding region is flanked by non-coding (NC) sequences. Each end can be divided into a conserved part, whose sequence is common to all the viral segments and specific for each type, and a non-conserved part. The length of this non-conserved sequence is very variable between segments and between influenza virus types. Within each genomic RNA molecule, the 3' and 5' ends associate to form two possible secondary structures: the 'panhandle structure' and the 'corkscrew structure'. These structures are known to be critical for the transcription and the replication of the vRNAs. Based on reverse genetic systems available for both type A and C influenza viruses, we attempted to rescue infectious viruses, for which one or both NC regions of the NP segment were exchanged between type A and C influenza viruses. This approach was used to demonstrate the importance of the panhandle structure in type specificity; more specifically of the proximal panhandle sequence and of the initial distal panhandle strength. Thus, both elements were found to be compulsory to rescue infectious viruses. Interestingly, in type A influenza virus infectious context, we also found that the length of the NP segment 5' NC region once transcribed into mRNA impacts its translation, and that the level of produced NP protein consequently affects the level of viral genome replication.

Keywords: non-coding, panhandle, nucleoprotein, type specificity, replication, translation

P11

The Location of the Acylation Site Relative to the Membrane Border is crucial for differential S-acylation of Influenza Virus Hemagglutinin

Katharina Brett[1], Larisa V. Kordyukova[2], Marina V. Serebryakova[2,3], Ramil R. Mintaev[2,4], Andrei V. Alexeevski[2,5] and Michael Veit[1]

[1]Institut für Virologie, Free University Berlin, 14163 Berlin, Germany
[2]A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia
[3]Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia
[4]I.I. Mechnikov Research Institute of Vaccines and Sera, Russian Academy of Medical Sciences, Moscow, Russia
[5]Department of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Moscow, Russia

Hemagglutinin (HA), the major glycoprotein of Influenza virus, is S-acylated at three conserved cysteines. This modification is essential for virus viability; promoting the opening of the fusion pore during viral cell entry and supporting budding of viruses by inclusion of HA into rafts. Our previous mass spectrometry analysis revealed differential attachment of acyl chains to HA. Stearate is exclusively attached to a cysteine at the end of the transmembrane region (TMR) whereas two cysteines in the cytoplasmic tail contain only palmitate.
Here we analyzed recombinant WSN virus containing HA with exchange of conserved amino acids adjacent to acylation sites or with a TMR cysteine shifted to a cytoplasmic location to identify the molecular signal that determines preferential attachment of stearate. We observed cell-type specific differences in HA's fatty acid pattern; more stearate was attached in human viruses grown in mammalian compared to avian cells. The mass spectra did not show underacylated peptides and mutations lethal for virus replication did not abolish acylation of expressed HA as shown by metabolic labeling experiments with 3H-palmitate. Substitution of conserved amino acids in the vicinity of an acylation site moderately affected the stearate content. In contrast, shifting the TMR cysteine to a cytoplasmic location virtually eliminated attachment of stearate. Hence, the location of an acylation site relative to the transmembrane span is the main signal for stearate attachment, while the sequence context and the cell type modulate the fatty acid pattern.

Keywords: Influenza virus; protein acylation; hemagglutinin; fatty acid; mass spectrometry

P12

Serological evidence of partial protection against infection with A/H3N2 circulating drifted strains after immunization with enhanced 2011/2012 trivalent influenza vaccines

Camilloni Barbara[1], Basileo Michela[1], Menculini Giuseppe[2], Tozzi Paolo[3], Iorio Anna Maria[1]

[1]Dept. Exp. Med. University of Perugia, Italy; [2]RP Bittoni, Città della Pieve, Perugia, Italy; [3]Azienda USL2, Foligno, Italy

A(H3N2) influenza viruses presenting antigenic and genetic patterns (A/Victoria/208-clade) different from the A(H3N2) vaccine component (A/Perth/16/2009-clade) were isolated during the 2011-2012 season in Europe. Results obtained using a test-negative case-control study design demonstrated a suboptimal protective effect of the vaccine especially in older people and it was postulated that this could be due to a combination of a poor match between vaccine and circulating viruses and a waning protection. Our results obtained using a different method support this hypothesis.
Ninety-two institutionalized elderly subjects were vaccinated with an influenza enhanced vaccine (Fluad® or Intanza® 15mcg) for the 2011-2012 season. Haemagglutination inhibiting (HI) antibody titers were determined in blood samples collected before, 1 and 6 months after vaccination against the three vaccine antigens and against four drifted A(H3N2) viruses circulating in the area where the elderly people were living. A serological diagnosis of influenza infection was made on comparing HI titers found in sera collected 1 and 6 months after vaccination. Patients were considered positive if they had a seroconversion.
No seroconversions were found against the A(H1N1) and B vaccine components and 6 months after vaccination HI titers decreased as compared with those found at one month. Instead, 17 (18.5%) volunteers seroconverted against A(H3N2) antigens. In most instances the infections were evidenced using both the vaccine and the epidemic A(H3N2) viruses as antigens. One month after vaccination, 15 of the 17 volunteers (88.2%) had post-vaccination seroprotective HI titers (≥40) against vaccine antigen, but not always against the epidemic strains.

Keywords: Influenza; Influenza vaccines; Homologous and heterologous response; Immunogenicity, Elderly

P13

Prevention of Lethal H1N1 and H7N9 Influenza Virus Infections by Novel Receptor Binding Proteins

H Connaris [1], EA Govorkova [2], Y Ligertwood [3], BM Dutia [3], L Yang [1], S Tauber [1], MA Taylor [1], N Alias [1], R Hagan [1], AA Nash [3], RW Webster [2] and GL Taylor [1]

[1] University of St. Andrews, St. Andrews, Scotland; [2] St. Jude Children's Research Hospital, Memphis, TN, USA; [3] The Roslin Institute, Edinburgh, Scotland.

A novel approach in combating influenza infection is to target host cellular receptors using protein-based biologics that recognize sialic acid (SA). This approach can prevent viral attachment during initial stages of infection and avoids drug resistance issues associated with virus-targeted therapies. Here we report the engineering, characterization of multivalent proteins based on SA-recognizing carbohydrate-binding modules (mCBMs) and their potential in preventing influenza virus infection. mCBMs were generated as oligomers using PCR-based cloning techniques using genes encoding the SA-binding domain from bacterial sialidases. Binding of mCBMs to SA and antiviral activity were explored *in vitro* against different influenza strains. mCBMs exhibited linkage-independent, nanomolar binding for SA. Mammalian cell protection was observed against several influenza virus strains (EC_{50} <4μM). *In vivo* efficacy of mCBMs was examined in BALB/c mice where mCBMs were intranasally administered as single or repeat doses up to 7d before or 24h after lethal challenge with A/WSN/1933(H1N1), A/California/2009(H1N1) or A/Anhui/1/2013(H7N9) viruses. mCBMs conferred protection against lethal doses of both H1N1 viruses and the H7N9 virus, with a hexameric mCBM demonstrating 100% survival when given as a single 1-μg (H1N1) or 10-μg (H7N9) dose 7d before challenge, with repeat dosing being the most beneficial regimen for complete protection. Moreover, anti-viral antibodies were detected in all treated mice. Furthermore, mCBMs stimulated the expression of pro-inflammatory mediators that may contribute towards their protective ability. Overall, a host-targeted approach using mCBMs can prevent influenza infection when administered repeatedly in advance of viral challenge. This approach could be extended to other SA-binding respiratory pathogens.

Keywords: genetic engineering, influenza, drug delivery, host-targeted

P14

Interference of influenza A virus with type I interferon-mediated signaling

Michel Crameri[1], Thomas Ludersdorfer[2], and Jovan Pavlovic[1]

[1]Institute of Medical Virology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
[2]Institute of Virology and Immunology (IVI), Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland

Type I interferons (IFNs) act as the first line of defense against viral infections. Induced by pattern recognition receptors in infected cells, they are secreted and bind to their cognate receptor in an autocrine or paracrine fashion. Thereby, they initiate the IFN-mediated signaling cascade that eventually leads to the establishment of an antiviral state owing to the production of IFN-induced effector proteins. Type I IFN-mediated signaling involves phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylated STATs heterodimerize and translocate to the nucleus, where they activate transcription of IFN-responsive genes.
Previous studies have shown that influenza A virus (IAV) disrupts type I IFN-mediated signaling. However, the mechanisms involved are currently only marginally characterized and remain a matter of dispute. In this study, we first investigated which viral factors are responsible for blocking the signaling response to IFN. Using a luciferase-based reporter system, we found that forced expression of the non-structural protein 1 (NS1) derived from an avian IAV strain showed a dramatic negative impact on type I IFN-induced gene expression. However, STAT1 phosphorylation did not appear to be affected in cells overexpressing NS1. At the same time, an avian IAV strain deficient for NS1 was still able to block IFN-induced gene expression. Here, we found that inhibition of the type I IFN-mediated signaling cascade occurred at a step before STAT1 phosphorylation.
Taken together, we suggest that influenza A viruses have evolved multiple strategies to inhibit type I IFN-mediated signaling, relying on both NS1-dependent and NS1-independent functions.

Keywords: NS1, interferon system, JAK-STAT signaling

P15

Infection of primary endometrium cells with influenza A (H3N2) virus can cause their functional dysregulation

Danilenko D.M.[1], Smirnova T.D.[1], Pisareva M.M.[1], Durnova A.O.[2], Kadirova R.A.[1], Smirnova S.S.[1]

[1]Research Institute of Influenza, Saint-Petersburg, Russia
[2]D.O. Ott Research Institute of Obsterics and Gynecology, Saint-Petersburg, Russia

Influenza infection in pregnant women can cause substantial harm not only for the mother but for the developing fetus as well. We investigated the influence of influenza A(H3N2) infection on the primary endometrium cells obtained on the 8th day of the cycle. Cells were infected with high (0.3) and low (0.0003) doses of A/Brisbane/10/07 virus. Cytopathic effect was very weak and could be observed only in cells infected with high MOI. The induction of apoptosis determined by Hoechst staining was also weak. However, endometrium cells infected with influenza virus, showed enhanced cellular proliferation which could be registered at least for three passages after initial infection. This effect was more pronounced in cells infected with low MOI. Viral RNA was detected by RT-PCR in all three passages of the cells infected with high dose of the virus, but was undetectable after the first passage in endometrium cells infected with low dose. High viral doses induced increased levels of Bcl-2, p-53, MMP-9 and TNF-α as determined by immunocytochemistry, whereas infection with low MOI caused induction of cell division proteins Ki-67 and cyclin A. Thus infection of primary endometrium cells with different MOI of influenza A(H3N2) virus causes functional changes in the activity of these cells. High viral doses lead to cell cycle arrest in G_0/G_1 phase, which is accompanied by TNF-α production and induction of MMP-9. Low viral doses stimulate cellular proliferation by induction of Ki-67 and cyclin A and simultaneous decrease of Bcl-2, p-53, MMP-9 and TNF-α.

Keywords: influenza, endometrium, cellular proliferation

P16

Serologic evidence of occupational risk for avian influenza virus transmission at wildfowl-poultry-human interface

Maria Alessandra De Marco[1], Mauro Delogu[2], Livia Di Trani[3], Arianna Boni[3], Matteo Frasnelli[4], Luciano Venturi[5], Fausto Marzadori[4], Isabella Donatelli[6], Elisabetta Raffini[4]

[1]Istituto Superiore per la Protezione e la Ricerca Ambientale, Ozzano Emilia (BO), Italy; [2]Department of Veterinary Medical Sciences, University of Bologna, Ozzano Emilia (BO), Italy; [3]Department SPVSA, Istituto Superiore di Sanità, Roma, Italy; [4]Istituto Zooprofilattico

Sperimentale della Lombardia e dell'Emilia Romagna, Lugo (RA), Italy; [5]Azienda Unità Sanitaria Locale di Ravenna, Ravenna, Italy; [6]Department MIPI, Istituto Superiore di Sanità, Roma, Italy

Low pathogenic (LP) avian influenza viruses (AIVs) can cause human infections, as the result of direct transmission from infected birds. Human cases are related to mild or subclinical disease, although severe outcomes have been recently reported in subjects infected by H7N9 LPAIVs. Some farming practises are modelled to house birds outdoor, thus exposing them to AIVs harboured in wild avian reservoir. These relatively low bio-security conditions enable the emergence of AIVs in captive-reared birds, increasing the potential zoonotic risk to occupationally exposed workers. Main purpose of this study is to provide serological evidence of LPAIV infection in workers operating in farms located in the Emilia-Romagna Region (Italy). In April 2005-July 2006 period, sera were collected from 57 bird-exposed workers and 7 non bird-exposed controls, planning three blood sample collections from each individual to assess seroconversion against AIVs. Study population included: 46 poultry workers (PWs) and 4 veterinarians operating in 14 farms housing birds under outdoor conditions; 2 veterinarians and 4 technicians involved in AIV laboratory diagnostics; a wildlife professional exposed to wild waterfowl.
Haemagglutination inhibition (HI) assay and microneutralization-ELISA (MN) were used as screening and confirmatory tests, respectively. Sera were tested for specific antibodies against twenty LPAIVs belonging to antigenic subtypes from H1 to H14 and including twelve reference strains and eight avian isolates from farms under study. MN results showed serologic evidence of exposures to AIV in 3/46 PWs, showing antibodies against H3, H6, H8 and H9 AIVs. In addition, the wildlife professional was seropositive against H11 AIV subtype.

Keywords: Poultry exposed workers; LPAIVs exposure; Wildlife-poultry-human interface; Human serosurvey; AIV human serology

P17

Comparison of innate immune responses to infection with swine influenza virus H1N1 in naïve pigs and pigs pre-infected with *Mycoplasma hyopneumoniae*

Deblanc C.[1,2], Delgado-Ortega M.[3,4], Berri M.[3,4], Herrler G.[5], Meurens F.[6], Simon G. [1,2]

[1]Anses, Laboratoire de Ploufragan-Plouzané, Unité Virologie et Immunologie Porcines, Ploufragan, France
[2]Université Européenne de Bretagne, France
[3]INRA, Infectiologie et Santé Publique, Nouzilly, France
[4]Université François Rabelais, UMR1282 Infectiologie et Santé Publique, Tours, France
[5]Institut für Virologie, Tierärztliche Hochschule Hannover, Hanover, Germany.
[6]Vaccine and Infectious Disease Organization-InterVac, University of Saskatchewan, Saskatchewan, Canada

Mycoplasma hyopneumoniae (Mhp) was previously shown to enhance clinical outcomes and pathogenesis of swine influenza virus H1N1 during the first week after H1N1 inoculation. The objectives here were to study and compare the innate immune responses that follow H1N1 infection in pigs pre-infected either with Mhp or not. Three groups of 9 SPF pigs were used: i) H1N1-inoculated at 9 weeks of age (H1N1 group); ii) Mhp-inoculated at 6 weeks of age and H1N1-inoculated 3 weeks later (MH1N1 group) and iii) mock-inoculated pigs (Control group). Three pigs from each group were sacrificed at 5, 24 and 48 hours post-H1N1 infection (hpi). Similar clinical signs were observed in both infected groups, except coughing that was reported in MH1N1 group only. The extent of macroscopic pulmonary lesions increased similarly over time and lesions scores were identical in both infected groups at 48hpi. Quantification of pathogens in lungs revealed similar amounts and distribution whatever the infected group. However, histological analyses showed earlier and more severe lung microscopic lesions in pigs pre-infected with Mhp, with an important influx of neutrophils and a strong inflammatory reaction. Concentrations of IL-6, IL-12, IFN-α and IFN-γ measured in BALF were similar in both infected groups at 5, 24 and 48 hpi H1N1, but TNF-α and IL-1β concentrations were higher at 5hpi in MH1N1 co-infected pigs. Quantification in lung tissues of several mRNA related to IFN response, cytokine production and regulation of innate response confirmed a higher inflammatory response in co-infected animals as compared to animals infected only with H1N1, and showed additive effects of both pathogens.

Keywords: swine influenza virus, coinfection, immune responses, inflammation

P18

Temperature-sensitive mutants in the influenza virus RNA-polymerase: the PA hinge domain is involved in the PB1-PA complex nuclear targeting and its mutagenesis can mediate attenuation

Bruno Da Costa[1], Alix Sausset[1], Nadia Naffakh[2,3,4], Ronan Le Goffic[1], Bernard Delmas[1]

[1]INRA, Unité de Virologie et Immunologie moléculaires, F-78350 Jouy-en-Josas
[2]Institut Pasteur, Unité de Génétique Moléculaire des Virus à ARN, Département de Virologie, F-75015 Paris
[3]CNRS, URA3015, F-75015 Paris
[4]Université Paris Diderot, Sorbonne Paris Cité, Unité de Génétique Moléculaire des Virus à ARN, F-75015 Paris

The influenza virus RNA-dependent RNA polymerase catalyses genome replication and transcription within the nucleus of infected cell. Efficient nuclear import and assembly of the polymerase subunits, PB1, PB2, and PA are limiting factors for the generation of reassortants. We investigated the structure and function of the PA hinge (residues 197-256), a domain located between its endonuclease and a large structured domain that binds the N-terminus of PB1, the polymerase core. A large series of PA hinge mutants exhibited a temperature-sensitive (*ts*) phenotype (at 39.5°C vs. 37°C/33°C), suggesting an alteration of folding kinetic parameters. A minireplicon assay showed the *ts*-phenotype was associated to a defect in the vRNA replication/transcription activities of the polymerase complexes (or any upstream steps preceding their assembly). Using a fluorescent-tagged PB1 subunit, we observed that PA *ts*- and lethal mutants do not efficiently recruit the PB1 subunit to reach the nucleus at 39.5°C. Several *ts*-mutants were shown attenuated and able to induce antibodies. Selection of revertants allowed identification of compensatory mutations in the three polymerase subunits. Recovery of a wild-type phenotype (loss of the replication temperature sensitivity and efficient PB1 targeting to the nucleus at 39.5°C) was observed in two PA reversion mutants. Taken together, our results identified a key flexible domain in PA as a "hot spot" to engineer numerous *ts*-mutants. This domain is critical to the nuclear import of PB1 and could be used to design attenuated vaccines and possibly antiviral drugs.

P19

Protection against influenza A virus challenge with M2e-displaying filamentous E. coli phage

Lei Deng1,2, Lorena Itati Ibañez1,2, Veronique Vandenbossche1,2, Kenny Roose1,2, Walter Fiers1,2, Xavier Saelens1,2

1 Inflammation Research Center, VIB, Technologiepark 927, Ghent, Belgium
2 Department of Biomedical Molecular Biology, Ghent University, Technologiepark 927, Ghent, Belgium

Influenza virus is responsible for annual epidemics causing severe illness approximately 5 million people worldwide. Matrix protein 2 (M2) of influenza A virus is a tetrameric type III membrane protein that functions as selective ion channel. The extracellular domain of M2 (M2e) remains almost conserved among human and avian influenza A virus. To develop an economical and easy to purify M2e vaccine, the M2e2-16 from a human H3N2 virus was genetically fused to the N-terminus of pVIII, the major coat protein of bacteriophage fd88. Intraperitoneal immunization with purified huM2e2-16-pVIIIfd88 phages, adjuvanted with incomplete Freund's adjuvant, induced robust M2e-specific serum IgG and protected BALB/c mice against challenge with human and avian influenza A virus challenge. Thus, presenting M2e immunogens on bacteriophage nanoparticles represents a promising candidate for developing an economical and effective universal influenza A vaccine.

Keywords: influenza A virus, Matrix protein 2, fd88 phage

P20

Combination therapy against influenza A virus infection

Rebecca Dewar[1], Sharon Tamir[2], Willie Donachie[3], Bernadette Dutia[1], Paul Digard[1]

[1]The Roslin Institute, The University of Edinburgh, Easter Bush, EH25 9RG
[2]Mercer Rd, Natick, MA 01760, United States
[3]Moredun Scientific, Pentlands Science Park, Penicuik, EH26 0PZ

Influenza A virus (IAV) causes substantial morbidity and mortality annually, especially in high-risk individuals such as the elderly and young children. Vaccination is the primary strategy to combat the transmission of influenza virus. However, the unpredictable antigenic changes in IAV strains can render vaccines ineffective. Consequently, antiviral drugs are critical to prevent transmission and severe illness caused by IAV. Currently, the U.S Food and Drug Administration only approve of two classes of antivirals against influenza virus; adamantanes and neuraminidase inhibitors, both of which IAV has developed resistance to. Combination treatment to avoid selection of resistant viruses has been a successful strategy for antiretroviral therapy against HIV and is a promising area of IAV antiviral research. The aim of this project is to develop a successful synergistic IAV therapy that will have a lower probability to develop viral resistance. The start of the project will focus on testing an array of compound combinations *in vitro* and determining which compounds are less likely to elicit viral drug resistance. The lead combinations will then be tested in a mouse model with the two most successful combinations further tested in ferrets, the gold standard animal model for IAV infection, in collaboration with Moredun Scientific.

Keywords: influenza A virus, antivirals, synergy, resistance.

P21

TRIM22 is an Interferon-β Dependent Intracellular Effector against Influenza A Virus Infection

Andrea Di Pietro*, Isabel Pagani & Elisa Vicenzi

Viral Pathogens and Biosafety Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy.
*presenting author

Members of Tripartite motif (TRIM) protein superfamily are emerging as important effectors of the innate immune intracellular response against viral infections. In particular, TRIM22 was reported to exert antiviral activity against RNA viruses, such as hepatitis B virus, encephalomyocarditis virus, human immunodeficiency virus type 1 and, as we have recently shown, Influenza A Virus (IAV) infection (Di Pietro et al., JVI, 2013). Since TRIM22 expression is both induced by IAV infection and type-I Interferon (IFN), we tested whether IAV-induced TRIM22 expression during infection was dependent on the production of endogenous type-I IFN.
A549 epithelial cells were infected with a high multiplicity of A/New Caledonia H1N1 (MOI=1) in co-treatment with different concentrations of either polyclonal anti-human-IFN-α or anti-human-IFN-β anti-sera. The quantification of TRIM22 and INF-β RNA was determined by real time PCR. IFN-β expression was upregulated up to 100-fold 4 h post-infection and was maintained at high levels after 12 h of infection whereas the kinetics of TRIM22 expression were delayed by 4 h as compared with those of IFN-β expression. Dilution of 1:30,000 and 1:25,000 of sheep anti-human-IFN-α and anti-human-INF-β sera inhibited 1,000 IU of IFN-α and 10 IU of IFN-β, respectively. Anti-IFN-β but not anti human IFN-α serum downregulated TRIM22 mRNA expression in A549 cells. In conclusion, TRIM22 is an INF-β stimulated gene that contributes to the antiviral response counteracting IAV infection.

Keywords: Influenza A virus, interferon, restriction factors, TRIM22

P22

Virus supportive function of the macroautophagy-related proteins Beclin1 and Atg7 during influenza A virus infection

S. E. Dudek*, and S. Ludwig

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

Macroautophagy is a cellular process, which directs cytoplasmic cellular components as well as invading pathogens to lysosomes for lysosomal degradation. On the induction of macroautophagy a complex interplay of different autophagy-related genes/ proteins (Atgs) takes place. For many viruses interplay with macroautophagy is documented, which may have virus-supportive as well as antiviral features.
Influenza A viruses (IAV) are well known to interact with many different proviral and antiviral cellular signaling pathways to ensure their replication. Recent publications report interplay of IAV with the macroautophagy machinery; however, a detailed picture of the influence on viral replication is still missing. Here we analyzed the impact of different autophagy-related genes (Atgs) on IAV life cycle. While a knock-down of Atg7 and Beclin1 leads to reduced viral titers in A549 cells, no differences were obtained when Atg5 or Atg12 were missing. Further, the knock-down of Atg7 and Beclin1 reduced IAV-induced type I IFN signaling in a stronger way compared to a lack of Atg5 and Atg12. However, we excluded a major role of type I IFN response in reducing viral titers since titers were also decreased in IFN-deficient Vero cells treated with Atg7 and Beclin1 siRNA. Further investigations revealed that the synthesis of viral mRNAs was not affected by the lack of Atg7 and Beclin1. Nevertheless, reduced viral protein accumulation was observed when Atg7 and Beclin1 were missing in A549 as well as Vero cells. We conclude that Atg7 and Beclin1 are functioning to support efficient IAV replication by interfering with viral protein accumulation.

Keywords: Beclin1, Atg7, proviral, viral protein accumulation

Note: Parts of these data were presented at different meetings before.

P23

Antiviral and cytoprotective activity of Influcid® in cell cultures model systems

M. Eropkin, E. Eropkina, T. Bryazhikova,

Research Institute of Influenza, Russian Ministry of Public Health, Saint-Petersburg, Russian Federation

The complex preparation Influcid® (Deutsche Homöopathie-Union) is used in the prophylaxis and treatment of some acute respiratory diseases including influenza. The purpose of this work was to study its possible direct or indirect antiviral effects in cell cultures against different respiratory viruses: adeno-, corona-, para-influenza, respiratory-cyncitial-viruses and also two types of herpes-viruses possibly connected to the secondary immune-deficits. The second purpose was to study a possible cytoprotective effect of Influcid® in the conditions of toxicity *in vitro* caused by high concentrations of such antiviral drugs as Rimantadine and Arbidol.
Earlier we have demonstrated antiviral activity of Influcid® *in vitro* against a set of actual seasonal and pandemic influenza strains of human and avian origin and a positive effect in mice model when challenged with lethal and sub-lethal doses of mice-adapted A/PR/8/34 strain.
Here we revealed the antiviral activity of the preparation against all studied respiratory viruses. Influcid® proved to be the most effective when introduced 6-24 h before virus inoculation (the *in vitro* model of "prophylactic" application). The powerful protective effect against Herpes simplex virus, especially of the type II, is very interesting in view of the postulated connection of the later with secondary immune-deficits.
Besides antiviral activity Influcid® exhibited a general cytoprotective effect in the conditions of toxic concentrations of Rimantadine and Arbidol which was evident in the MTT-test. Hereby, we could conclude that the antiviral activity of Influcid® is to a considerable degree caused by a non-specific cytoprotective activity against the viral cytopathogenic effect.

P24

The study of HA stalk-directed heterosubtypic antibody responses against Influenza A viruses elicited by seasonal vaccination using a pseudotype neutralization assay

Francesca Ferrara[1], Eleonora Molesti[1], Eva Böttcher-Friebertshäuser[2], Davide Corti[3], Emanuele Montomoli[4] and Nigel Temperton[1]

[1]Viral Pseudotype Unit, School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, ME4 4TB, United Kingdom
[2]Institute of Virology, Philipps University Marburg, Hans-Meerwein-Str. 2 ,35043 Marburg, Germany
[3]Institute for Research in Biomedicine, Via Vela 6, 6500 Bellinzona, Switzerland
[4]Department of Molecular and Developmental Medicine, University of Siena, Via Aldo Moro 3, 53100 Siena, Italy

The evaluation of the ability of vaccines to elicit heterosubtypic antibody responses to confer broad protection against different influenza subtypes is an important facet of pandemic preparedness. Classical serological assays, such as haemagglutination inhibition and microneutralization, have demonstrated low sensitivity for the detection of cross-neutralizing antibodies, especially those directed against epitopes in the haemagglutinin (HA) stalk region. Influenza pseudotypes represent safe tools to study the neutralizing antibody response since they are replication-defective viruses and they harbour on their envelope only the HA that is the major target of this response. We have generated a panel of human, avian and chimeric (head-stalk) influenza A pseudotypes and have employed them as surrogate antigens in neutralization assays to study the presence and magnitude of heterosubtypic neutralizing antibody responses in human sera collected before and after routine 2007-2008 seasonal influenza vaccination.
In the human sera tested using a pseudotype neutralization assay, heterosubtypic neutralizing antibody responses are detected and the neutralizing antibody end-point titres are boosted after seasonal vaccination. Using chimeric HA pseudotypes as antigen in the neutralization assay, the results show that the heterosubtypic antibody responses detected are principally directed against the HA stalk region. The increased sensitivity of the pseudotype neutralization assay performed using a panel of influenza A pseudotypes permits the detection of HA stalk-directed heterosubtypic antibody responses before and after seasonal influenza vaccination.

Keywords: heterosubtypic, antibody response, pseudotypes, serology

P25

Production of Influenza B haemagglutinin lentiviral pseudotype particles and their use in neutralization assays

Francesca Ferrara[1], Eva Böttcher-Friebertshäuser[2], Stefan Pöhlmann[3], Davide Corti[4], Paul Kellam[5], Sarah C Gilbert[6] and Nigel Temperton[1]

[1]Viral Pseudotype Unit, School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, ME4 4TB, United Kingdom
[2]Institute of Virology, Philipps University Marburg, Hans-Meerwein-Str. 2, 35043 Marburg, Germany
[3]Infection Biology Unit, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany
[4]Institute for Research in Biomedicine, Via Vela 6, 6500 Bellinzona, Switzerland
[5]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, CB10 1SA, United Kingdom
[6]The Jenner Institute, Old Road Campus Research Building, Roosevelt Drive, University of Oxford, Oxford, United Kingdom.

Influenza B (FluB) viruses cause respiratory disease epidemics in human populations. Furthermore an Influenza B strain is routinely included in the seasonal vaccine and recently a quadrivalent vaccine containing two FluB strains was developed. Serological methods permit the evaluation of Influenza epidemiological distribution and are additionally used to evaluate vaccine efficacy. However the haemagglutination inhibition assay has been shown to be relatively insensitive for the detection of antibodies against FluB viruses. The use of replication-defective viruses, such as pseudotypes, in microneutralization assays is an accepted and safe alternative approach to study antibody responses elicited by natural infection or vaccination. We have produced a panel of Influenza B haemagglutinin (HA) pseudotypes using plasmid-directed transfection. To activate FluB HA we have explored the use of proteases by adding the relevant encoding plasmid to the transfection mixture. When tested for their ability to transduce target cells, the FluB pseudotypes exhibit high transduction titers. With these newly developed reagents, we are investigating the use of FluB pseudotypes as surrogate antigens in neutralization assays on human trial sera.

Keywords: Influenza B virus, antibody response, protease, pseudotype serology

P26

The study of protease-mediated haemagglutinin activation using influenza A virus pseudotypes.

Francesca Ferrara[1], Eleonora Molesti[1], Eva Böttcher-Friebertshäuser[2], Stefan Pöhlmann[3], Davide Corti[4], Giovanni Cattoli[5], Simon Scott[1] and Nigel Temperton[1]

[1]Viral Pseudotype Unit, School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, ME4 4TB, United Kingdom
[2]Institute of Virology, Philipps University Marburg, Hans-Meerwein-Str. 2, 35043 Marburg, Germany
[3]Infection Biology Unit, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany
[4]Institute for Research in Biomedicine, Via Vela 6, 6500 Bellinzona, Switzerland
[5]Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 Legnaro, Padua, Italy

The monomer of influenza haemagglutinin (HA) is synthesized as a polypeptide precursor that during maturation is cleaved by proteases into two active subunits. Recent studies have demonstrated the role of the human transmembrane protease serine 2 (TMPRSS2), the human transmembrane protease serine 4 (TMPRSS4) and the human airway trypsin-like protease (HAT) in HA activation by cleavage. As a model of haemagglutinin activation we have used influenza HA lentiviral pseudotypes. Influenza pseudotypes were obtained by co-transfecting human embryonic kidney HEK293T/17 cells using plasmids coding for the influenza HA, HIV gag-pol and a lentiviral vector incorporating firefly luciferase. In order to investigate the role of proteases in HA activation, a plasmid expressing protease was co-transfected during pseudotype production. The influenza pseudotypes produced in the presence of proteases were then tested for their ability to transduce HEK293T/17 cells and were compared to pseudotypes produced in the absence of proteases, and after trypsin treatment to mediate HA cleavage and activation.
The results obtained show that TMPRSS2, TMPRSS4 and HAT can activate, *in vitro*, with differential specificity both the HA of human seasonal influenza and also other avian HA influenza strains in a pseudotype production system.

Keywords: haemagglutinin, cleavage, protease, pseudotypes

P27

Back-boost of antibody landscapes after influenza virus infection and vaccination

J. M. Fonville[1,2], S. H. Wilks[1,2], S. L. James[1,2], M. Aban[3], L. Xue[3], T. C. Jones[1,2], M. Ventresca[1,2], A. Fox[4], Le N. M. H.[4], Pham Q. T.[5], Tran N. D.[5], Y. Wong[1,2?], A. Mosterin[1,2], L. C. Katzelnick[1,2], G. van der Net[6], E. Skepner[1,2], C.A. Russell[1,2], T. D. Kaplan[7], G. F. Rimmelzwaan[6], N. Masurel[6†], J. C. de Jong[6], A. Palache[8], W. E. P. Beyer[6], Le Q. M.[5], Nguyen T. H.[5], H. Wertheim[4], A. Hurt[2], A. D. M. E. Osterhaus[6], I.G. Barr[3], R. A. M. Fouchier[6], P. Horby[4], D. J. Smith[1,2,6]

[1]Center for Pathogen Evolution, Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK. [2]WHO Collaborating Center for Modeling, Evolution, and Control of Emerging Infectious Diseases, Cambridge CB2 3EJ, UK. [3]WHO Collaborating Centre for Reference and Research on Influenza, Melbourne VIC 3051 Australia. [4]Oxford University Clinical Research Unit and Wellcome Trust Major Overseas Programme, Hanoi, Vietnam. [5]National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. [6]Department of Virology, Erasmus Medical Center, Rotterdam 3015 CE, the Netherlands. [7]Todd's affiliation. [8]Abbott Laboratories, Weesp 1380 DA, the Netherlands.
[†]Professor Masurel is deceased

We introduce the *antibody landscape*, a method for quantitative visualization and analysis of antibody-mediated immunity to antigenically variable pathogens, achieved by accounting for antigenic variation among pathogen strains. We generated antibody landscapes based on 43 years of influenza A/H3N2 virus evolution for 69 individuals followed for six years, and pre- and post-vaccination landscapes for 225 individuals. Upon infection and vaccination, we found a surprising *back-boost* – an increase of antibodies across much of an individual's prior subtype-specific immunity. We explored this phenomenon for vaccine design, and found that vaccinating with an antigenically advanced vaccine virus had the dual benefit of inducing antibodies against the advanced antigenic cluster, and against previous clusters. These results indicate that pre-emptive vaccine updates may substantially improve influenza vaccine efficacy in previously-exposed individuals.

Keywords: Vaccination, infection, antibody, modelling.

P28

Attenuation of MxA-resistant Influenza Viruses is due to impaired nuclear vRNP import

Veronika Götz[1], Linda Magar[1], Dominik Dornfeld[1], Byung-Whi Kong[2], Bhuwan Khatri[2], Martin Schwemmle[1]

[1]Institute for Virology, University of Freiburg, 79104 Freiburg, Germany;
[2]Center of Excellence for Poultry Science, University of Arkansas Fayetteville, AR 72701, USA

Three amino acids in the nucleoprotein (NP) are necessary to escape the antiviral activity of the human restriction factor MxA. Viruses circulating in the human population carry these amino acids, whereas avian strains including H5N1 viruses harbor different amino acids in these positions, rendering them MxA-sensitive. Substitution of these amino acids in the NP of human pandemic H1N1 (pH1N1) viruses with the corresponding amino acids of H5N1 MxA-sensitive viruses, leads to an increase in viral replication compared to wt pH1N1 in MDCK cells. Vice versa, introduction of MxA-resistance enhancing amino acids into an H5N1 strain (H5N1-3X) attenuates viral replication. This suggests that acquisition of MxA-resistance is accompanied by impaired viral fitness. This was further supported by the identification of escape mutants (H5N1-3X-esc) that lost MxA-resistance and grew to higher titers compared to H5N1-3X. Primer extension analysis in the presence of cycloheximide revealed significantly lower levels of primary transcripts in H5N1-3X-infected cells compared to cells infected with wt H5N1. Cell fractionation and immunofluorescence experiments demonstrated that this impaired primary transcription is due to a block in nuclear import of infecting vRNPs. Attenuation of H5N1-3X was observed in all tested cell lines including avian and mammalian cells. Because of striking differences, we hypothesize that a host cell factor is needed for efficient nuclear import of vRNPs and that vRNPs harboring MxA-resistance enhancing amino acids cannot recruit this factor efficiently. In summary we show that gaining MxA resistance is accompanied by impaired viral fitness due to impaired nuclear import of vRNPs.

P29

Antiviral effects of PI3K-gamma during influenza A infection

Cristiana Couto Garcia, Luciana Pádua Tavares, Ana Carolina Fialho Dias, Celso Queiroz Junior, Braulio Henrique Freire Lima, Alexandre Magalhães Vieira Machado, Lirlândia Pires De Sousa, Remo Castro Russo, Mauro Martins Teixeira

PI3Kγ, mainly expressed by leukocytes, is activated by GPCRs and involved in cell migration during inflammation. Influenza A virus causes a common and severe pulmonary disease that affects millions of people worldwide every year. We assessed for the first time the specific role of PI3Kγ during influenza A infection. PI3Kγ KO mice were more susceptible to WSN and PR8 infection, with enhanced lethality and weight loss rates than WT mice. The enhanced susceptibility in KO during infection was correlated to higher neutrophil infiltration, ROS production in lungs and lung damage and lower IL-10 release and number of resolving macrophages. On the other hand, antiviral response – Type I and III IFN expression, p38 phosphorilation, NK and CD8+ T cells infiltration – were strikingly reduced in KO mice, while viral loads were increased. Surprisingly, ISG15, Mx2 and ddx58 expression was highly enhanced in KO mice, whereas protein levels of ISG15 and RIG-I were similar in WT and KO. Similar results were found in BMDM but not fibroblasts ex vivo infection, where blockade of P-p38 reduces ISG15 protein in WT cells, similarly to KO infected cells. We demonstrated that PI3Kγ is necessary for effective antiviral responses and resolution of inflammation induced by influenza A infection. PI3Kγ KO induces an IFN-independent induction of ISGs RNA not translated into protein. Therefore, PI3Kγ via p38 might be involved in postracriptional regulation of ISGs.

Financial support: CNPq/CAPES/FAPEMIG

Keywords: PI3Kγ, type I IFN, inflammation, influenza

P30

The interaction network between the human PR8 (H1N1) influenza A virus genomic RNA segments

Marie Gerber[1], Vincent Moules[2,3], Matthieu Yver[2], Manuel Rosa-Calatrava[2], Roland Marquet[1] and Catherine Isel[1]

[1]Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084 Strasbourg, [2]Virologie et pathologie Humaine, Université de Lyon 1, Faculté de Médecine RTH Laennec, 69008 Lyon, [3]VirNext, EA4610, Faculté de Médecine RTH Laennec, 69008 Lyon

Influenza A viruses (IAVs) are characterized by a segmented genome consisting of 8 single stranded viral RNAs (vRNA) of negative polarity. Each vRNA is associated with the viral polymerase complex and covered by the nucleoprotein to form a viral ribonucleoparticle (vRNP).
To be infectious, viral particles must contain at least one copy of each vRNP. Data from our group[1,2] and others[3] strongly support the existence of a selective packaging mechanism involving *cis*-acting bipartite signals on all vRNAs of IAVs. Results obtained in the laboratory on a human H3N2 and an avian H5N2 virus suggest that the 8 vRNPs are selected and packaged via a supramolecular complex maintained by vRNA/vRNA interactions. Importantly, some of the interactions identified *in vitro* were proven to be crucial for viral replication and packaging[4].
Surprisingly, the interaction networks and the sequences involved were different for the two viruses. In order to investigate a potential host and/or subtype specificity, we are now focusing on the human A/Puerto Rico/8/34 (H1N1) (PR8) virus, used for the production of the seasonal flu vaccines. First, we defined, by band-shift assays, the PR8 interaction network. Interestingly, it is different again from the H3N2 and H5N2 ones. The interacting sequences are being investigated using a combination of deletion mutagenesis, antisense oligonucleotide mapping and bio-informatics.
The ultimate goal of our project is to unveil the molecular mechanisms and the potential rules underlying the packaging of the genome of IAVs and in turn to better understand the clearly biased genetic reassortment process.

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Keywords: Influenzavirus A, Packaging, Viral RNA, RNA/RNA interaction

P31

Antiviral activity of modified antisense oligonucleotides against Influenza virus H1N1.

Goncharova E.*, Kupryushkin M., Stetsenko D., Pyshny D., Zenkova M.

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of Russian Academy of Sciences, Russia

Taking in account that influenza virus is a real threat to human health, development of new antiviral therapeutics is urgently needed. Here we tested a panel of modified antisense oligonucleotides (AON) targeting the translation start site region of PB1 and the terminal region of NP of influenza virus mRNA. The cytotoxicity of compounds was tested in MTT-test. Antiviral activities of AONs were evaluated on MDCK cells. Cells were mock (IMDM medium)-treated or treated with ON with lipofectamine for 4 h in serum-free medium. The cells were then inoculated with virus at an m.o.i. of 0.01 for 60 min. After incubation, virus was removed and the cells were incubated in fresh IMDM. At time point 24h post-infection, supernatant was collected and virus titers were determined on MDCK cell by in focus form unit assay. The obtained results demonstrated moderate antiviral activity of modified AON target the terminal region of NP of influenza virus mRNA. Further studies are ongoing to evaluate the inhibitory activity of novel panel of AON potently inhibiting virus replication in MDCK cells.

Keywords: influenza, antiviral, antisense oligonucleotides

P32

Avian influenza viruses H4N6 subtypes isolated from Caspian seals, Russia.

M.A.Gulyaeva[a], A.Yu.Alekseev[b], M.V.Sivay[b], V.A.Kuznetsov[c],S.V. Shipulin[c], A.M.Shestopalov[b]

a Novosibirsk State University, Novosibirsk, 630090, Russia
b Research Center of Clinical and Experimental Medicine, Siberian Branch of Russian Academy of Medical Sciences, Novosibirsk, 630117, Russia
c Federal State Unitary Enterprise Caspian Fisheries Research Institute, 414056, Astrakhan, Russia

Influenza is an acute infectious disease of the respiratory tract caused by influenza viruses. Influenza provokes annual incidence rises, frequent epidemics and periodic pandemics.
Avian influenza viruses (AIV) penetrating from the natural hosts reservoirs are the most pathogenic, and infect different animal species including pigs, marine mammals and birds. AIV may occasionally causes devastating pandemic in a human population. Interspecies transmission also occurs between humans and animals, which are not natural hosts of these viruses. It is supposed that the reason of an influenza pandemic is the penetration of new virus subtypes or some of its genes from animal populations. As a result, a high level of danger for human.
The purpose of the research is to study influenza virus in reservoirs which are investigated less. The result of surveillance in Caspian seals in 2002 and 2012 is isolation and identification of 2 AIV isolates. The interest is to study and to compare molecular-biological characteristics of isolated viruses. During the study we found out that these isolates belong to H4N6, a low pathogenic avian influenza virus subtype. All segments of viral genome were sequenced. Amino acid and phylogenetic analyzes were conducted as well. According the results, differences and similarities in the viruses were detected. Amino acid analysis also showed differences between strains isolated from seals and all H4N6 isolated from birds. Biological properties of H4N6 subtype viruses were studied in mice.

Keywords: Influenza, Caspian seal, virus, biological characteristics, sequencing

P33

Host SUMOylation responses to influenza A virus infection

P.S. Domingues[1,*], F. Golebiowski[1,*], A. Taggart[1], M.H. Tatham[2], R.T. Hay[2] and B.G. Hale[1]

[1]MRC – University of Glasgow Centre for Virus Research, UK
[2]Centre for Gene Regulation and Expression, University of Dundee, UK
[*]These authors contributed equally

Dynamic protein modification by the ubiquitin-like modifier SUMO contributes to an array of nuclear biology, notably cellular stress responses. Here, we characterize the host SUMOylation response to influenza A virus (IAV), observing that infection causes a dramatic redistribution of intra-nuclear SUMO which parallels a global increase in SUMO conjugates. IAV-induced SUMOylation is dependent on virus genome replication and protein synthesis, but not RNP nuclear export, and viral polymerase activity may promote this response. Nevertheless, cell-based analysis of virus-activated stress pathways showed that increased SUMOylation with IAV is independent of canonical MAVS-, IRF3- and STAT1- mediated innate immunity, and does not correlate with activation of unfolded protein, apoptotic, PKR or DNA damage pathways.

Using affinity-based SILAC proteomics, we identified the SUMO1- and SUMO2- modified proteome of human lung epithelial cells, and quantified specific changes to SUMOylation during virus infection. Strikingly, only 130 cellular proteins increase significantly in SUMO1/2 modification with IAV compared to the bulk of other cellular SUMO substrates (765 proteins). Results from shRNA-depletion screening of 80 IAV-induced SUMO substrates suggested that IAV infection re-targets SUMO to several novel host factors involved in restriction of virus replication, as well as to components of the human PAF1 complex known to regulate antiviral gene expression. Bioinformatic studies also revealed that IAV-triggered SUMOylation is largely distinct from other SUMO responses to stress stimuli such as heat shock, ionizing radiation and bacterial infection. Overall, our global survey of SUMO modifications identifies a unique virus-triggered cellular SUMO response that may modulate aspects of host immunity.

Keywords: nucleus, SUMO, proteomics, stress, host response

P34

The alpha-inducible protein 27 like 2A (Ifi27l2a) is active in lung macrophages and neutrophils after influenza A infection but is not induced by interferon in epithelial cells

Bastian Hatesuer[a], Mohamed A. Tantawy[a,b], Esther Wilk[a], Nadine Kasnitz[c], Siegfried Weiß[c], and Klaus Schughart[a,b,d]

[a]Department of Infection Genetics, Helmholtz Centre for Infection Research, [b]University of Veterinary Medicine Hannover, [c]Department of Molecular Immunology, Helmholtz Centre for Infection Research and [d]University of Tennessee Health Science Center

The interferon pathway represents one of the most important host defense mechanism against viral infections. Subsequent induction of both type I and type III interferons by infected cells is a hallmark of the early response to infection. Upon secretion and binding to specific receptors these proteins stimulate intracellular and intercellular networks that regulate transcriptional activation of hundreds of interferon-activated genes, also referred to as ISG (interferon-stimulated) or IFI (interferon induced) genes.
The alpha-inducible protein 27 like 2A (*Ifi27l2a*) gene is strongly up-regulated in the lung after influenza A infection in mice and has been shown in gene expression studies to be highly correlated to other activated genes.
Here, we investigated the expression and function of the IFN-induced *Ifi27l2a* gene during the course of influenza A virus infection. We describe the cell type-specific expression of *Ifi27l2a* in the lung and its role for the host defense to influenza A infections in *Ifi27l2a* knock-out mice. In the lungs of non-infected animals *Ifi27l2a* was strongly expressed in macrophages and also found in lymphocytes. After influenza infection a strong increase in overall expression levels of *Ifi27l2a* was observed in the lung. This increase was mainly due to infiltration of macrophages and lymphocytes and not traces back to an expression in epithelial cells near infected cells. We thus conclude that the increase observed for *Ifi27l2a* transcripts in the lung after infection is mainly due to infiltration of *Ifi27l2a* expressing macrophages and lymphocytes but not up-regulation of *Ifi27l2a* in individual cells.

Keywords: Ifi27l2a mutant mice, Interferon pathway, macrophages

P35

Influenza Virus Infection Dynamics in the Elderly

Esteban A. Hernandez-Vargas[1], Esther Wilk[2], Laetitia Canini[3], Franklin R. Toapanta[4], Sebastian C. Binder[7], Alexey Uvarovskii[7], Ted M. Ross[5], Carlos A. Guzmán[6], Alan S. Perelson[3], Michael Meyer-Hermann[7],[8]

[1]Systems Medicine of Infectious Diseases and Braunschweig Integrated Centre of Systems Biology, Helmholtz Centre for Infection Research, Inhoffenstr. 7, Braunschweig, 38124, Germany
[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, New Mexico, USA
[4]Center for Vaccine Development, University of Maryland, Baltimore, Maryland, USA
[5]Center for Vaccine Research, University of Pittsburgh, Pittsburgh, Pennsylvania, USA
[6]Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany
[7]Department of Systems Immunology and Braunschweig Integrated Centre of Systems Biology, Helmholtz Centre for Infection Research, Inhoffenstr.7, Braunschweig, 38124, Germany
[8]Institute for Biochemistry, Biotechnology and Bioinformatics, Technische Universität Braunschweig, Braunschweig, Germany

Elderly individuals (>65 years of age) are affected by influenza infections more often and usually with more severe symptoms which last longer than in adults. A weakened immune response, allowing the infection to prosper, has been proposed as one of the main factors responsible for these phenomena; however, there is still much debate about other factors that can be playing a role.
While natural killer cells and antigen-specific CD8+ T cells are important in the immune response to influenza, our mathematical models suggest the level of inflammatory cytokines in elderly mice may play a critical role. Such cytokines, which reach higher levels in aged mice, may cause host target cells in elderly mice to become more resistant to the virus than the host cells of younger mice. Due to this influenza virus may replicate more slowly in elderly mice such that their immune system may recognize the infection too late and may not be sufficiently triggered to generate an efficient response. Some of the cytokines responsible for this effect are also present at higher basal concentrations in elderly people, a situation called "inflamm-aging". This result, if applicable to elderly people, would shed a different light on the reasons for less efficient control of viral infections in the elderly population.

Reference: Hernandez-Vargas E. A., Wilk, E., Canini, L., Toapanta F., Binder S., Uvarovskii A., Ross T., Guzman C. A., Perelson A. S., Meyer Hermann M. 2014. The effects of aging on influenza virus infection dynamics. Journal of Virology: 88 (14)

P36

Deletion of interferon regulatory factor 3 (*Irf3*) in mice leads to delayed immune response and higher lethality after influenza infection

Hang T.T. Hoang[1], Bastian Hatesuer[1] and Klaus Schughart[1,2,3]

1 Department of Infection Genetics, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany
2 University of Veterinary Medicine Hannover
3 University of Tennessee Health Science Center, Memphis

Activation of the interferon pathway is one of essential mechanisms of the host defense that inhibit spread and replication of viral pathogens and further trigger additional innate immune responses. Interferon regulatory factor 3 (*Irf3)* was previously reported to play a crucial role in the innate immunity against certain bacteria and viruses. In this study, a mouse model carrying an *Irf3* null allele was characterized after infection with PR8 H1N1 influenza virus. Although body weight loss and survival showed a moderately susceptible phenotype in comparison with wildtype C57BL/6J mice, we observed a delay in the onset of the host innate immune responses. This was reflected by distinct expression of cytokines, chemokines and interferon stimulated genes (ISGs) at the mRNA level and a significant higher viral load at early times post infection. These initial data re-emphasized the hypothesis of both an early contribution of *Irf3* to a rapid host defense and a later compensation by alternative interferon activating systems that control virus replication and allow the host to survive.

Keywords: cytokines, influenza A, interferon stimulated genes, Irf3, knock-out mouse

P37

2009 pandemic H1N1 influenza A virus mortality is associated with an impaired innate and cellular immune response during pregnancy in mice

Julia Hoffmann[1*], René Thieme[2*], Géraldine Engels[1*], Petra Arck[2] and Gülsah Gabriel[1]

[1]Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany
[2]Department of Obstetrics and Fetal Medicine, Laboratory for Experimental Feto-Maternal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[*]equally contributed

Pregnancy is considered a main risk factor for influenza related illness and death. This has been particularly highlighted during the most recent H1N1 influenza pandemic in 2009. Subsequently, vaccine recommendations have been revised worldwide and the World Health Organization now recommends vaccination of pregnant women with first priority. Although the enhanced risk to develop severe illness has been related to several physiological changes such as hormonal alterations during pregnancy, the underlying molecular mechanisms still remain elusive. In order to analyze the molecular pathways involved in increased H1N1 pandemic influenza pathogenesis during pregnancy, we have established a semiallogeneic pregnant mouse infection model that mirrors the clinical findings in humans. Here, we show that 2009 H1N1 pandemic influenza virus infection during pregnancy leads to a generally reduced cytokine response and dendritic cell activation that is further accompanied by an impaired ability of $CT8^+$ T-cells to clear viral infection. These findings suggest that innate and adaptive immune pathways undergo a tolerogenic state during pregnancy that causes a contradictory demand for the host immune system to eliminate viral infection. Finally, insights from research on pregnancy-associated risk for the development of severe influenza may increase awareness and improve the influenza vaccination compliance of women in their reproductive years or during pregnancy.

Keywords: influenza, pregnancy, mouse model, innate immune response, adaptive immune response

P38

Reverse genetics as a tool to characterize resistance mechanisms of nucleoprotein inhibitors (NPI).

Hoffmann Anja[a], Von Grafenstein Susanne[b], Liedl Klaus R.[b], Rollinger Judith M.[c], Sauerbrei Andreas[a], Schmidtke Michaela[a]

[a]Department of Virology and Antiviral Therapy, Jena University Hospital, Hans-Knoell-Straße 2, 07745 Jena, Germany, [b]Institute of General, Inorganic and Theoretical Chemistry, Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria; [c]Institute of Pharmacy, Department of Pharmacognosy, Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria

The highly conserved influenza nucleoprotein is essential for viral replication and therefore represents a valid new drug target. Recently, nucleozin was found to inhibit viral replication by interaction with nucleoprotein. Amongst human pathogenic influenza, some H1N1 and H3N2 strains show resistance to nucleozin and analogs. Especially the mutation Y289H present in A(H1N1)pdm09 strains confers distinct resistance against NPI. In this study we characterize resistance profiles of chlornucleozin analogs. Reverse genetics were used to study how these compounds could overcome nucleozin resistance.
Initially, we compared susceptibility profiles of H3N2 and pandemic H1N1 represented by A/Hongkong/1/68 (HK/68) and A/Jena/8178/09, respectively. In addition, we generated recombinant variants of HK/68 and the H1N1 influenza virus A/WSN/1933 (WSN/33). For each strain two variants with known nucleozin resistance-conferring mutations in the nucleoprotein (Y52H; Y289H) were rescued from eight plasmids. The susceptibility of the engineered variants against all NPI was compared to respective nucleozin-susceptible wild type strains and the naturally resistant pandemic H1N1.
It was found that several novel NPI were able to inhibit the pandemic H1N1, but not the equivalent WSN/33 with the Y289H mutation. Likewise, against the Y52H variant no inhibitory effect could be detected. An additional mutation (NPY313V) was introduced to overcome the discrepancies.
Taken together, our results demonstrate the possibility to overcome the resistance by substitutions at the chlornucleozin scaffold. Nevertheless our findings also show that breaking NPI resistance within one genetic background does not guarantee effective inhibition amongst different strains.
This work is supported by the ESF (2011-FGR0137) and the FWF (P23051,P24587).

Keywords: nucleoprotein, resistance, inhibitors, reverse genetics, chlornucleozin

P39

Detection of Virus Genome on Animal Surfaces and in Litter in the Course of Outbreaks with Low-Pathogenic Avian Influenza

Timo Homeier[1], Timm Harder[2], Yvonne Gall[1], Franz Conraths[1]

[1]Institute of Epidemiology, [2]Institute of Diagnostic Virology of the Friedrich-Loeffler-Institut

Infection with low pathogenicity avian influenza (LPAI) viruses is widespread in the European Union (EU). Outbreaks of LPAI (H5 or H7) in poultry are often controlled by culling affected flocks to avoid a further spread of the disease. However, derogation from the depopulation principle is possible if a risk assessment comes to the conclusion that the risk of further spread is minimal.
Little is known about the distribution of LPAI viruses within flocks, the extent of environmental contamination and the tenacity of the virus under field conditions. This gap in knowledge also leads to uncertainties in risk assessments.
The aim of the study was to determine the extent of LPAIV contamination of litter and animal surfaces (feathers, feet) in the case of an LPAI outbreak. In total, 415 samples of litter, feathers and feet were collected immediately after culling of three LPAI (H7N7) infected turkey herds. In addition, 180 combined tracheal and cloacal swabs were taken from the turkeys. All samples were diagnosed by H7 specific RT-PCR.
The highest proportions of positive samples were found in litter (up to 85.9%). The results for the combined swabs were similar, while the percentage of positive foot and feather samples was lower (up to 18.3% or 5.0 %, respectively).
There is substantial contamination of litter and to a lesser extent also of the animal surface with LPAI virus genome. Slaughtering such animals and processing them for human consumption conventionally can therefore not be recommended. Further investigations are needed to determine the persistence of contaminating influenza virus and its infectivity.

P40

Influenza virus infection induced several cellular changings: sialic acid and proteins of endosome.

Yukari MIYAKE[1], Jaebum CHO[2], Takahiro KIMURA[1], Madoka TAKAI[2] and Ayae HONDA[1]

1 Frontier Bioscience, Hosei University
2 Department of Engineering, Graduate school of engineering, University of Tokyo

The genome of influenza virus is composed of eight negative strand RNAs, which form vRNP (RNA, RNA-dependent RNA polymerase and NP). Eight vRNPs are wrapped in a membrane derived from host cell. On the membrane there are heamaglutinin (HA) and neuraminidase (NA). Influenza virus binds sialic acid on the cell membrane through HA and enters into cytoplasm by endocytosis. We thought that the influenza virus infection induced some changes to the cell, which are the expression level of endosomal proteins and sialic acid. To understand the changing of the expression of endosomal proteins, we assayed the content of α-adaptin, β-adaptin and AP50. To assay sialic acid content of the cell, we used the fluorescence-labeled lectin for ･2,3- and ･2,6-linked sialic acid, and sialic acid content in the cell was assayed using sialic acid quantitation kit. Here, we show the results that influenza virus infection reduced sialic acid level of the cell and the level of ･･-adaptin. We will discuss further at the meeting.

P41

E172K in NS1 Promotes Adaptation of the novel H7N9 virus to a Mammalian Host.

Xiaofeng Huang, Siu-Ying Lau, Min Zheng, Pui Wang, Wenjun Song, Siwen Liu, Bobo Mok, Honglin Chen

State Key Laboratory for Emerging Infectious Diseases and Department of Microbiology, the University of Hong Kong

Cross species transmissions of H5N1, H9N2 and H7N7 subtype avian influenza viruses have occurred sporadically in recent years. However, the rapidity and magnitude of the emergence of human infections with H7N9 since the spring of 2013 is particularly alarming. What are the unique features of this H7N9 avian influenza A virus? Studying the mammalian adaptation strategies of this virus will give us clues for understanding the requirements for cross species transmission and efficient human transmission. Besides the relatively well characterized adaptive changes of receptor specificity for binding human cells associated with the hemagglutinin (HA) protein and PB2 polymerase of these subtypes of avian influenza A virus, another less well defined area is adaptive changes to virus replication in the NS1 protein. The amino acid residue 172E is highly conserved in NS1 protein encoded by influenza A viruses. However, all the newly emerged H7N9 viruses carry E172K substitution. We found the E172K substitution significantly enhance H7N9 replication in A549, a human lung adenocsarcinoma epithelial cell line, but not in DF-1, a chicken fibroblast cell line. This substitution showed no effect on the interferon induction, viral RNA replication and viral mRNA transcription during one single cycle infection. Further characterization found that E172K substitution is important for virus adaptation to the mammalian cell intrinsic antiviral immunity and supporting virus replication in the mammalian cells.

P42

Development of a spray freeze-dried whole inactivated virus influenza vaccine for pulmonary administration

Harshad P. Patil[a], Senthil Murugappan[b], Jacqueline de Vries- Idema[a], Tjarko Meijerhof[a], Jan Wilschut[a], , Henderik W. Frijlink[b], Wouter L.J. Hinrichs[b], Anke Huckriede[a*]

a Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.
b Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.

Current inactivated influenza vaccines are still suboptimal: a.o. they have to be administered by tedious intramuscular injection, they are highly vulnerable to degradation when not stored within a very tight temperature range (2-8^{o}), and they fail to elicit immunity in the nose and throat and thus do not prevent early stages of the infection.
To overcome these drawbacks we converted liquid vaccine to a dry powder and delivered this powder vaccine directly to the lungs (pulmonary immunization). Dry powder vaccines produced by spray freeze-drying of the antigens with suitable sugars as stabilizer were stable at temperatures of 30°C for at least 3 months. Two administrations of these powder vaccines to the lungs of mice elicited influenza specific immunity comparable to that achieved by conventional intramuscular injection. The immune responses could be further improved and IgA and IgG titres in the airways could be enhanced by including suitable adjuvants in the vaccine. Mice that received two pulmonal immunizations with dry powder vaccine adjuvanted with the saponin-derived compound GPI-0100 mounted the strongest systemic and mucosal antibody responses and were partially protected against challenge with a heterologous virus strain.
Our findings demonstrate that formulation of influenza vaccines as stable dry powders is feasible and imply that these vaccines will elicit strong local and systemic immunity when administered to the lungs e.g. by simple inhalation. The stability of the vaccine and the ease of administration makes dry powder vaccines especially suitable for use in resource-poor countries and for mass vaccination campaigns as required in case of pandemics.

P43

N-Glycosylation of Influenza Virus Hemagglutinin Impacts Immunogenicity

J. Hütter[1,2], J. Rödig[3], D. Höper[4], U. Reichl[3], P.H. Seeberger[1,2], E. Rapp[3], B. Lepenies*[1,2]

[1]Max Planck Institute of Colloids and Interfaces, Department of Biomolecular Systems and [2]Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin
[3]Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Sandtorstr. 1, 39106 Magdeburg
[4]Friedrich-Loeffler-Institut (FLI), Südufer 10, 17493 Greifswald - Insel Riems, Germany

Hemagglutinin (HA) is the most abundant glycoprotein on the surface of the influenza virus and represents a major virulence factor. Most vaccines against influenza target HA to elicit protective immunity. Influenza vaccine manufacturing using mammalian cell lines represents a promising alternative to conventional virus propagation in embryonated chicken eggs. Besides virus strain and subtype, also the host cell line used for influenza virus production determines HA *N*-glycosylation. Due to an increasing demand for cell-line based influenza vaccines it is of special interest in which way differential protein glycosylation affects virus immunogenicity.
In this study, we investigated the impact of the differential HA *N*-glycosylation patterns of two influenza A virus PR/8/34 (H1N1) variants on immunogenicity. Next-generation pyro-sequencing validated the congruence of the potential HA *N*-glycosylation sites. For stimulation studies, cells from TCR-HA transgenic mice were used. T cells from these mice express a TCRαβ specific for the $HA_{110–120}$ peptide presented by I-E^d MHC class II molecules. *In vitro* stimulation and further *in vivo* immunization studies with MDCK cell-derived and Vero cell-derived influenza virus preparations revealed a significant difference in the immunogenicity of the two glycovariants. Furthermore, natively deglycosylated virus preparations induced dramatically reduced cellular and humoral responses.
In conclusion, we show that influenza A virus PR/8/34 (H1N1) HA *N*-glycosylation markedly affects immunogenicity. The platform presented here allows for a rapid screening of HA *N*-glycosylation patterns and directly correlates them with immunogenicity. Hence, appropriate host cell lines can be selected for efficient virus vaccine production.

Keywords: hemagglutinin, *N*-glycosylation, immunogenicity, vaccine production

P44

Therapeutic potential of murine bone marrow derived mesenchymal stem cells in influenza virus-induced pneumonia

Lina Jankauskaite, Carole Schmoldt, Jürgen Lohmeyer, Susanne Herold

University of Giessen and Marburg Lung Center, Department of Internal Medicine, Justus Liebig University, Klinikstrasse 33, 35392 Giessen

Influenza virus (IV) infects the human upper respiratory tract and occasionally spreads to the alveolar compartment causing primary pneumonia. This can lead to acute respiratory distress syndrome (ARDS) with severe alveolar damage, lung oedema and hypoxemia. Antiviral therapies are only effective in the very beginning of infection and specific treatment strategies for IV-induced ARDS are lacking. Recent studies have shown the anti-inflammatory and regenerative potential of mesenchymal stem cells (MSC). MSC display a beneficial role in acute and chronic lung injury, suggesting that MSC delivery may be a promising treatment strategy in IV-induced ARDS [1, 2].
We isolated MSC cells from the bone marrow of 8 to 12 weeks old C57Bl6 mice by cell sorting [3]. We characterised their expression markers by flow cytometry and we confirmed their differentiation potential into chondrocytes, osteocytes and adipocytes by microscopy. We co-cultured primary murine alveolar epithelial cells (AECs) with sorted MSC. During influenza infection with PR8 strain, the presence of MSC drastically reduced apoptosis and infection level in AECs. We tested the effect of MSC intratracheal instillation in PR8-infected mice. Similarly to the *in vitro* experiments, the addition of MSC improved clinical outcomes in comparison with 3T3/PBS-instilled control mice.
Our experiments show the beneficial role of MSC in PR8-induced injury in vitro and in vivo. Taken together our results support that MSC can be of great value to treat IV-induced lung injury.

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2. Gupta N. et al. 2012. Mesenchymal stem cells enhance survival and bacterial clearance in murine Escherichia coli pneumonia. Thorax,67(6):533-9.

3. Houlihan D.D. et al. 2012. Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-α. Nat protoc,7(12):2103-11.

Keywords: Influenza virus, acute respiratory distress syndrome, mesenchymal stem cells

P45

Rational design and evaluation of bio-shields for control of avian influenza virus

S. Jasim, L. Vervelde, A.A. Nash, B.M. Dutia, P. Digard, M.P. Stevens

The Roslin Institute & Royal (Dick) School of Veterinary Studies, University of Edinburgh

Avian influenza viruses can cause devastating outbreaks in domesticated poultry, with rapid transmission of virus between birds and high mortality. Current measures for the control of influenza in reservoir hosts involve culling of infected animals and vaccines which are costly, labour-intensive and require seasonal updating. While it is reported that the introduction of increased biosecurity measures in poultry wet markets has reduced the spectrum of influenza subtypes, the threat of zoonosis is still apparent, as evidenced by the transfer of H7N9 and H10N8 to humans earlier last year in China. Studies thus far have evaluated the protective effects of two anti-viral peptide families; 'FluPep' (a series of short hydrophobic peptides related to the SOCS3 mimetic) and 'Entry Blocker' (derived from the signal sequence of fibroblast growth factor-4). Anti-viral activity, assessed by plaque reduction assays, was observed for FP1, FP4, and B10NP peptides against a panel of low pathogenic avian influenza viruses within the nanomolar range. Ongoing work investigates peptide mechanism of action and findings suggest that they exert their inhibitory effects at the early stages of viral entry. Further work investigates the potential of engineering a common constituent of the indigenous microflora, *Lactobacillus*, to express and secrete these peptides for the development of prophylactic 'bio-shields' in chickens.

Keywords: Avian influenza, anti-viral peptide, FluPep

P46

Flt3 signaling is critical for host response after Influenza A infection

Nora Kühn[1], Friederike Schäkel[1], Bastian Hatesuer[1], Leonie Dengler[1], Esther Wilk[1], Marina Pils[2], Bruce Beutler[3], Klaus Schughart[1]

[1]Department of Infection Genetics, Helmholtz Centre for Infection Research, University of Veterinary Medicine Hannover, and University of Tennessee Health Science Center, 38124 Braunschweig, Germany
[2]Mousepathology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany
[3]Department of Genetics, The Scripps Research Institute, La Jolla, CA 92037, United States

FMS-like tyrosine kinase 3 (Flt3) is a hematopoietic receptor tyrosine kinase important for the development of many immune cells, including dendritic cells (DC) and natural killer (NK) cells. The *Flt3*$^{wmfl/wmfl}$ (*warmflash*) mouse mutant strain was identified in an ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis screen for susceptibility to cytomegalovirus (MCMV).
Flt3$^{wmfl/wmfl}$ mice are smaller than wild type of same age and sex. Flow cytometry analysis of lung cell populations indicates a decreased CD11c^{high} MHCII^{high} subpopulation, representing alveolar DCs in uninfected *Flt3*$^{wmfl/wmfl}$ lungs. Additionally a reduced NKp46^{+} cell population (NK cells) was found compared to wild type mice.
Flt3$^{wmfl/wmfl}$ mutants showed an increased susceptibility to influenza A virus infection compared to wild type mice. After infection with low virulent H1N1 PR8M virus *Flt3*$^{wmfl/wmfl}$ mice lost weight rapidly and died between 6 and 8 days after infection. In contrast, wild type controls regained body weight after day 7 and survived. At days 1 to 5 after infection *Flt3*$^{wmfl/wmfl}$ mice exhibited a similar viral load in the lungs as wild type mice. *Flt3*$^{wmfl/wmfl}$ responded to influenza A infection with an higher granulocyte count in peripheral blood compared to wild type mice. The analysis of immune cell infiltrates in the lung is ongoing.
In summary, the absence of Flt3 signaling in mice results in high susceptibility to influenza A infections. We hypothesize that a defect in the link between innate and adaptive immune response due to reduced numbers of DCs and NK cells in *Flt3*$^{wmfl/wmfl}$ mice is responsible for increased susceptibility to influenza virus infection.

Keywords: Flt3, murine influenza infection model, host response, dendritic cells

P47

Cholesterol and related host factors in influenza A virus infection

Alexander Kühnl,[1] Agnes Musiol,[1] Christina Ehrhardt,[2] Stephan Ludwig,[2] Volker Gerke,[1] Ursula Rescher[1]

[1]Institute of Medical Biochemistry, Centre for Molecular Biology of Inflammation and Interdisciplinary Clinical Research Centre, University of Münster, Münster, Germany;
[2]Institute of Molecular Virology, Centre for Molecular Biology of Inflammation, University of Münster, Münster, Germany

Influenza A virus (IAV) is the causing agent of influenza, a disease that represents an annual challenge to the public health worldwide. In search of antiviral targets, host cell factors came recently into focus. For this purpose comprehensive knowledge about the underlying mechanisms for pathogen-host-interaction is crucial.
Through its lifecycle IAV needs to overcome and interact with different cellular membranes. In this project we focus on host cell factors that are involved in these processes. Results from our previous work show that IAV replication depends on maintenance of the cellular cholesterol balance and identified the Ca^{2+}-regulated membrane-binding protein annexin A6 (AnxA6) as a critical factor in linking IAV to cellular cholesterol homeostasis. This was demonstrated for avian (H7N7) and human prototype (H1N1, PR8) IAV strains, as well as a strain derived from the 2009 pandemic swine-origin IAV.
Here, we examined the functional role of late endosomal cholesterol accumulation in IAV (H1N1, PR8) entry. We show that shifting of cellular cholesterol distribution either by AnxA6 over-expression or the use of a pharmacological agent, U18666A, reduces amounts of newly synthesized viral RNA and proteins within the first cycle of virus-replication. Using fluorescence microscopy we followed the IAV nucleoprotein and found that its nuclear localization is decreased in treated cells. Our further work will aim to elucidate which steps of the viral entry process are affected and how this eventually can be used in antiviral strategies.

P48

Activation of the interferon induction cascade by influenza A viruses requires viral RNA synthesis and nuclear export.

Marian Killip, Matt Smith, David Jackson & Richard Randall

University of St Andrews, St Andrews, Fife, UK

We have examined the requirements for virus transcription and replication, and thus the roles of input and progeny genomes, in the induction of interferon (IFN) by influenza A viruses using inhibitors of these processes. In the presence of cycloheximide or NP siRNA, which inhibit viral protein synthesis and thus cRNP and progeny vRNP synthesis, strong activation of the IFN induction cascade occurred in A549 cells infected with a range of influenza A viruses. In contrast, activation of the IFN induction cascade was very effectively abrogated by treatment with actinomycin D and other transcription inhibitors, which correlated with the inhibition of the synthesis of all viral RNA species. Furthermore, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole, an inhibitor that prevents viral RNA export from the nucleus, was also a potent inhibitor of IRF3 activation; thus, both viral RNA synthesis and nuclear export are required for IFN induction by influenza A viruses. Whilst the exact nature of the viral PAMPs remains to be determined, our data suggests that in this experimental system, the major influenza A virus PAMPs are distinct from incoming genomes or progeny vRNPs.

Keywords: interferon, influenza, IRF3, PAMP

P49

Lipoteichoic acid of *Staphylococcus aureus* as main contributor to the enhancement of influenza A virus induced mitogen-activated protein kinase signaling

Carolin Klemm[1], Kathrin Warnking[1], Bettina Löffler[2], Georg Peters[2], Stephan Ludwig[1], Christina Ehrhardt[1]

[1]Institute of Molecular Virology (IMV), Center for Molecular Biology of Inflammation (ZMBE), Westfaelische Wilhelms-University Muenster, Von Esmarch-Str. 56, D-48149 Muenster, Germany
[2]Institute of Medical Microbiology, University Hospital of Muenster, Domagkstr. 10, D-48149 Muenster, Germany

Bacterial co-infections are a major complication of influenza A virus (IAV) infections leading to severe illness. Recent findings suggest that beside the pathogen load, a dysregulated immune response of the host also contributes to increased morbidity. Several *in vivo* studies demonstrate elevated levels of cytokines and chemokines upon IAV and bacterial co-infections resulting in a massive influx of immune cells into the lung and severe tissue damage. Nonetheless, the underlying molecular signaling mechanisms still remain to be elucidated. However, this knowledge is crucial for development of new therapeutic approaches.
In this study we focused on cellular signaling mechanisms in a human lung epithelial cell line (A549) resulting in a dysregulated innate immune response upon co-infection with IAV and *Staphylococcus aureus* (*S. aureus*) *in vitro*. We observed elevated levels of cytokines and chemokines in a co-infection situation as described in *in vivo* models. Analyses of cellular signaling mechanisms regulating these innate immune responses genes revealed significantly increased activation of the mitogen-activated protein kinases (MAPKs) JNK and p38 in presence of both pathogens compared to IAV-infected cells. Similar results were obtained, when IAV infection was replaced with viral RNA or *S. aureus* infection was restored by lipoteichoic acid (LTA) stimulation, but not with other bacterial components.
Our data indicate a correlation of hyper-activation of MAPKs and overexpression of pro-inflammatory cytokines and chemokines. We will provide deeper insights in the regulation of pathogenicity during IAV and *S. aureus* co-infections on a molecular level, which contributes to the lethal synergism of these pathogens.

Keywords: influenza, *Staphylococcus aureus*, co-infection, MAPK signaling

Note: Parts of these data were presented at different meetings before.

P50

Interferon-λ plays an important role in influenza A virus spread from the upper respiratory tract to the lung and seems to reduce virus transmission in the mouse model

Jonas Klinkhammer[1,2], Tanel Mahlakõiv[1] and Peter Staeheli[1]

[1]Institute for Virology, University Medical Center Freiburg, 79104 Freiburg, Germany
[2]MOTI-VATE Promotionskolleg der medizinischen Fakultät Freiburg

Due to the lack of a suitable small animal model, host factors determining transmission of influenza A viruses are only poorly characterized. Ferrets and guinea pigs are frequently used to study virus-encoded traits which determine influenza virus transmissibility. However, they are not suitable for studying the role of host immune factors, such as virus-induced interferon (IFN), as there are no transgenic or knockout animals available. By contrast, although many genetically modified mice are available, influenza virus transmission is usually not observed between mice.
We isolated a variant of the Udorn influenza A virus strain which is readily transmitted upon contact among mice that lack functional receptors for both IFN-α/β and IFN-λ. If applied to the upper respiratory tract, this virus variant replicates well in the nose of wild-type mice but does not spread efficiently to the lung and is not found at high concentrations in the saliva. By contrast, in mice lacking functional receptors for IFN-λ, the virus quickly reaches the lung under the same experimental conditions and is abundantly present in the saliva. Preliminary data show that IFN-λ receptor-deficient mice transmit the virus more efficiently to cage mates upon direct cohousing than wild-type mice, indicating that IFN-λ represents an important host factor which limits influenza virus transmission in mice.

Keywords: Influenza A, mouse model, interferon-λ, upper respiratory tract, transmission

P51

Influenza surveillance in swine in North-Western region of Russia

Komissarov A.B[1]., Konovalova N.I.[1]*, Fadeev A. V[1]., Sintsova K. S[1]., Palaziuk S. V.[2], Aliev A. S.[2], Danilenko D.M.[1], Eropkin M.Yu.[1], Grudinin M. P.[1]

[1]Research Institute of Influenza, Saint-Petersburg, Russia
[2]State Academy of Veterinary Medicine, Saint-Petersburg, Russia

Emergence of a new pandemic strain of H1N1 subtype in 2009 has led to its subsequent introduction into swine population. In order to evaluate the evolutionary variability of A(H1N1)pdm09 in swine herds in Russia we performed screening of several swine farms located in close vicinity to Saint-Petersburg, Russia. 98 nasal swabs were collected and 7 of them were influenza A positive in RT-PCR. 5 of them were subtyped as H1N1pdm09 and 2 as H3N2 virus. Isolation of viruses in MDCK and chicken embryos permitted to retrieve 3 isolates, all of them were A(H1N1)pdm09. Antigenic analysis of these isolates showed that they were similar to human vaccine strain A/California/07/09 and were related to classical swine influenza viruses and old H1N1 viruses such as A/swine/Iowa/15/1930, A/swine/USA/1976/1931 and A/swine/HK/1/1974/.These results were confirmed by antigenic mapping of the isolates. Full genome sequencing revealed that all isolates were closely related to early human pandemic strains of 2009-2010, which is consistent with slower evolution of influenza A viruses in swine compared to humans. No reassortment in these swine isolates could be detected.

Keywords: swine influenza, A(H1N1)pdm09, genome sequencing

P52

The Non-structural protein 1 of influenza A virus interacts with the transcription factor C/EBP beta and downregulates RIG I promoter activity during viral infection

Rashmi Kumari[a] ,Shivaprakash Gangappa[b], Jacqueline M. Katz[b], Nancy J. Cox[b], Renu B. Lal[b], Paul B. Fisher[c], Takashi Fujita[d], Suryaprakash Sambhara[b], Priya Ranjan[b*] & Sunil K. Lal[a*]

[a]Virology Group, International Centre for Genetic Engineering & Biotechnology, New Delhi 110067, India
[b]Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA
[c]VCU Institute of Molecular Medicine, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298
[d]Laboratory of Molecular Genetics, Institute for Virus Research, Kyoto University, Kyoto, Japan
[*]to whom correspondence should be addressed

Innate immunity is the first line of host defence, which relies on several families of pathogen recognition receptors (PRR). The Retinoic acid inducible gene-I (RIG-I), a cytosolic PRR that recognizes influenza viral genome, is known to be inhibited by the viral non-structural protein 1 (NS1). Although post-translational regulation of RIG-I protein is well documented, its transcriptional regulation during influenza infection has not been established. Here, we investigated the regulation of the RIG-I promoter using both synthetic ligand 5'PPP containing ssRNAstimulation, as well as influenza virus infection. We identified the transcription factor, C/EBPβ as a novel regulator of the RIG-I promoter in the presence of 5'PPP-ssRNA. While 5'PPP-ssRNA failed to up-regulate RIG-I protein levels in cells over-expressing different isoforms of C/EBPβ, the down regulation of C/EBPβ with specific siRNA, enhanced the expression of the RIG-I gene. In addition, influenza virus infection also resulted in a significant increase in the binding of C/EBPβ to RIG-I promoter. To address the mechanism involved in the regulation of the RIG-I promoter by C/EBFβ during influenza virus infection, the role of NS1, a major virulence factor of influenza virus, was assessed by measuring the levels of C/EBPβ protein. In the presence of NS1, we observed increased levels of C/EBPβ protein as well as its MAPK-dependent phosphorylation. Further, our co-immunoprecipitation studies revealed an interaction between NS1 and C/EBPβ. Thus, the present study strongly suggests a novel role for C/EBPβ and NS1 protein in transcriptional down-regulation of the RIG-I gene during influenza virus infection.

Keywords: Transcriptional regulation, Virus-Host Interactions, Anti-viral host response

P53

Alteration of Protein Levels during Influenza Virus H1N1 Infection in Host Cells: A Proteomic Survey of Host and Virus reveals differential Dynamics

Susann Kummer *[1,2], Max Flöttmann*[1], Björn Schwannhäuser[3], Christian Sieben[2], Michael Veit[4], Matthias Selbach[3], Edda Klipp[1], Andreas Herrmann[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences I, Humboldt University Berlin, Berlin, Germany
[2]Department of Infectious Diseases, Faculty of Medicine, University Hospital Heidelberg, Heidelberg, Germany
[3]Max Delbrück Center for Molecular Medicine, Berlin, Germany
[4]Institute of Virology, Department of Veterinary Medicine, Berlin, Germany
*contributed equally to this work

Background: Invasive hijacking of the host cell machinery during viral infection is associated with directed influence on the host gene expression. Systematic approaches analyzing the host and the viral proteomic changes over the period of the entire viral replication process give new insights into virus-host-interactions. Previous studies addressing this issue found various host genes being essential for the influenza A virus infection cycle. However, it remained open how modification of gene expression translates into dynamics of the proteome and regulatory networks.
Method: We studied the dynamics of the proteome of influenza virus A (H1N1) infected cells up to 12 hours post infection by mass spectrometry based quantitative proteomics using stable isotope labeling of amino acids in cell culture (SILAC) in combination with bioinformatics processing of the obtained data set. For analysis we used functional clustering, gene ontology, and pathway (KEGG) enrichment tests to uncover co-regulated cellular protein sets, assigned the individual subsets to their biological function, and determined their interrelation within the progression of viral infection. For the first time, we are able to describe dynamic changes of the cellular and, of note, the viral proteome in a time dependent manner simultaneously.
Conclusion: Time dependent patterns of protein abundances revealed highly dynamic up- and/or down-regulation processes. This strongly suggests that there is an active influence on the host protein levels by viral infection.

P54

Swine IFITM proteins protect against influenza A virus infection *in vitro*

Caroline Lanz, Emilio Yángüez and Silke Stertz

The zoonotic potential of swine influenza viruses is a major threat to human health and causes huge economical losses, as could be seen during the swine-origin influenza pandemic in 2009. The antiviral potential of interferon-induced transmembrane proteins (IFITMs) against a wide range of viruses, including influenza A virus (IAV), has been described for human IFITMs. However, little is known about the expression and the antiviral activity of IFITM proteins present in swine. To close this gap, we examined and confirmed the presence and the induction of swine IFITMs upon interferon stimulation or infection in several porcine cell lines using qPCR. We then cloned the swine homologs of IFITM1, IFITM2, IFITM3 and IFITM5 and stably introduced them into newborn pig trachea epithelial cells (NPTr). When infecting these cell lines with human, avian or swine IAV strains we observed a significant reduction in viral titers compared to control cells. Furthermore, we tested the antiviral restriction capacity of swine IFITM proteins via a mini-genome reporter assay in NPTr and HEK293T cells and could confirm results obtained in the viral titer experiments. Localization studies revealed distinct patterns for the different IFITM proteins with either prominent plasma-membrane accumulation for swIFITM1a and 1b or an endosome-type distribution for swIFITM2, 3 and 5. In sum, we cloned and characterized the swine IFITM proteins regarding their localization, antiviral activity and interferon inducibility.

P55

Signaling molecules of the innate immune system as genetic adjuvants in DNA immunizations against Influenza A viruses

Dennis Lapuente, Michael Storcksdieck genannt Bonsmann, Viktoria Stab, André Maaske, Thomas Niezold, Christina Ehrhardt, Matthias Tenbusch

Introduction: Trivalent inactivated Influenza vaccines only confer strain-specific protection by inducing antibodies against the viral surface proteins of the vaccine strains and therefore require a yearly administration. In contrast, individuals who have overcome an Influenza infection develop partially a heterosubtypic immunity most probably due to cellular responses against conserved epitopes. The sensing of IAV components by pattern recognition receptors (PRR) plays a key role in the initiation of these broad acting immune responses.
Objectives: We hypothesized that coexpression of signaling molecules involved in the PRR-activated pathways can mimic an infection in DNA immunizations and thereby enhance the heterosubtypic immunity.
Methods: Balb/c mice were immunized via intramuscular injection of 30 µg DNA followed by electroporation. The vaccine included plasmids coding for Hemagglutinin and Nucleoprotein as well as for one of the adjuvant candidates RIG-I, IPS1, IL-1β or IL-18. Humoral and cellular responses were characterized. The efficacy was analyzed by homologous and heterologous IAV infections.
Results: Immunizations with antigen-encoding plasmids alone induced robust humoral and cellular responses resulting in complete protection against the homologous and partial protection against a heterologous virus infection. The coexpression of IL-18 seemed to increase antigen-specific T cell responses, whereas IL-1β had a significant influence on humoral responses mostly apparent as a shift of specific antibodies towards IgG1. In the heterologous challenge experiments animals vaccinated with either of these adjuvants showed by trend less weight loss during infection but the viral loads were not affected.
Keywords: Influenza, universal vaccine, DNA vaccine, genetic adjuvants, heterologous immunity

P56

Mathematical modeling of virus-host cell interactions during influenza A virus replication supports cell line development for vaccine production

Tanja Laske[1*], Frank Stefan Heldt[2], Timo Frensing[2], Udo Reichl[1,2]

[1] Otto von Guericke University, Chair of Bioprocess Engineering, Magdeburg, Germany
[2] Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Magdeburg, Germany
*Correspondence: laske@mpi-magdeburg.mpg.de

As an obligate intracellular parasite, influenza A viruses require a suitable host cell for reproduction. To compensate for its small genomic capacity the virus hijacks the metabolic and protein synthesis machinery of the cell in order to complete its replication cycle. Recently, genome-wide RNAi screens elucidated cellular factors involved in various pathways that are relevant for influenza virus replication. Our aim is to manipulate the expression of these factors in order to increase the virus yield in cell culture-based influenza vaccine production. For this we employ a systems biology approach to better understand the underlying influenza virus-host cell interactions.
In a previous work, our group established a detailed mathematical model which describes the intracellular life cycle of influenza A virus at the single cell level. Using this model we were able to identify key steps of the viral life cycle that represent bottlenecks during virus replication. In particular, our analysis showed that changes in the regulation of viral RNA synthesis can enhance virus growth. By contrast, the manipulation of steps involved in virus entry, transport processes and the assembly of viral genome complexes seems to have only minimal effects on final virus titres in bioreactors. Furthermore, model modifications enabled us to capture published experimental data on changes in viral RNA levels upon perturbation of host cell factors.
Overall, our approach provides an innovative, model-based strategy for the optimization of cell culture-based vaccine production processes and contributes to an improved understanding of virus-host cell interactions.

Keywords: influenza A virus replication, virus-host cell interaction, cell line engineering, influenza vaccine production

P57

Differential influenza A (H3N2) virus and bacteria-induced cytokine responses in blood leukocytes predict respiratory and gastrointestinal tract infection susceptibility in healthy military conscripts

Liisa Lehtoranta[1,2], Sinikka Latvala[4], Kim Kalima[1,3], Mikael Maksimow[5], Mika Mäkelä[6,3], Marko Salmi[5], Riitta Korpela[2], Simo Siitonen[1], Anne Pitkäranta[3, 7], and Ilkka Julkunen[4,8]

[1]Centre for Military Medicine, Research and Development Department, Finnish Defense Forces, Helsinki, Finland. [2]Institute of Biomedicine, Pharmacology, University of Helsinki, Helsinki, Finland. [3]Institute of Clinical Medicine, University of Helsinki, Helsinki, Finland.[4]National Institute for Health and Welfare, Helsinki, Finland, [5]MediCity Research Laboratory and Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland. [6]Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland. [7]Department of Otorhinolaryngology-Head and Neck Surgery, Helsinki University Central Hospital, Helsinki, Finland. [8]Department of Virology, University of Turku, Turku, Finland

In the present study we have analyzed whether there are differences in the cytokine production capacities between infection-prone and healthy military conscripts. Peripheral blood mononuclear cells (PBMCs) collected at the beginning of military service were stimulated with influenza A virus (InfA, H3N2), *Streptococcus pyogenes* (GAS) or *Escherichia coli* LPS and their microbe-induced cytokine production capacity was compared between healthy conscripts and those who suffered from repeated respiratory and gastrointestinal tract infections. A vast array of Th1, Th2, pro- and anti-inflammatory cytokines, chemokines, and growth and differentiation factors were analyzed. The magnitude and variety of cytokines/growth factors induced by InfA virus or LPS remained somewhat lower compared with GAS-stimulation. As expected, InfA stimulation induced higher IFN-‧2 and IP-10 production than GAS or LPS stimulation. The growth factor, colony-stimulating factor and proinflammatory cytokine responses remained weak in InfA-stimulated cells with the exception of TNF-‧, IFN-‧ and IL-2 which were expressed at moderate levels. The production levels of IL-15 and M-CSF in InfA-stimulated PBMCs were significantly higher in infection prone conscripts. The work also showed that in GAS-stimulated PBMCs certain proinflammatory cytokine levels including IL-1α, IL-1β, IL-18, and IL-12p40 were significantly lower in infection-prone conscripts compared with healthy recruits. This suggests that there is a relative difference in the ability of microbial stimuli to enhance inflammatory responses in favor of those that remained healthy during their military service. The data indicates that certain phenotypic characteristics of the cytokine production network in basically healthy individuals are associated with susceptibility to common microbial infections.

Keywords: Influenza, leukocyte, cytokine, chemokine

P58

Structural and functional characterization of α2-3-sialylated gangliosides as potential influenza A virus receptors of MDCKII cells

N. Legros[1], G. Pohlentz[1], C. Ehrhardt[2], S. Ludwig[2], H. Karch[1], J. Müthing[1]

[1]Institute for Hygiene, University of Münster, Münster, Germany
[2]Institute of Molecular Virology, ZMBE, University of Münster, Münster, Germany

Influenza A viruses bind to terminally sialylated glycan chains exposed on the surface of target cells. Gangliosides of the neolacto-series with a terminal Neu5Acα2-3Galβ1-4GlcNAc-epitope are supposed to preferentially bind to animal type influenza viruses, whereas the Neu5Acα2-6Galβ1-4GlcNAc-motif is believed to represent the preferred receptor oligosaccharide for human influenza viruses. Moreover, functional virus receptors with lipid membrane anchors such as gangliosides are expected to be associated with lipid rafts – dynamic membrane microdomains enriched in cholesterol, glycosphingolipids (GSLs), and/or sphingomyelin. Here we report on the identification of terminally sialylated tetrahexosylceramides of the neolacto series (neolactotetraosylceramide, nLc4Cer) with Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer core structure expressed by the MDCKII (Madin Darby Canine Kidney) cell line, which represents a certain subtype of the MDCK cell line. Gangliosides carrying *N*-acetylneuraminic acid (Neu5Ac) in α2-3-configuration at the terminal galactose were identified as potential virus receptors using immunochemical techniques combined with electrospray ionization (ESI) mass spectrometry (MS), whereas α2-6-sialylated gangliosides were undetectable in MDCKII cells. This finding suggests enhanced susceptibility of MDCKII cells toward animal influenza A viruses with preferential binding to glycoconjugates with Neu5Acα2-3-Galß1-4GlcNAc-terminus. Virus receptor gangliosides were found to be tightly associated with lipid rafts together with cholesterol and sphingomyelin, indirectly determined by their appearance in detergent-resistant membranes (DRMs), which were prepared by sucrose density gradient ultracentrifugation from MDCKII cells. Collectively, our results suggest that association of virus receptor gangliosides with lipid rafts may play an important functional role in binding and internalization processes of virions.

Keywords: Neolacto series gangliosides, sialic acids, lipid rafts, mass spectrometry

P59

Detecting respiratory viral pathogens isolated in pre-epidemic influenza season 2011 in Ukraine

Leibenko L. [1], Radchenko L. [1], Onishchenko O. [1], Golubka O. [1], Fesenko A.[1], Babii S. [1], Smutko O. [1], Mironenko A. [1]

[1]L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases" NAMS of Ukraine 5, Amosova Str., Kyiv, Ukraine, 03680

Multiplex real-time RT-PCR assays have been developed for simultaneous detection and identification of majority respiratory pathogens. The aim of our study was to perform a multiplex Real-Time PCR analysis of respiratory samples collected in pre-epidemic influenza season in Ukraine and analyze its effectiveness and feasibility.
16 nasal-throat swabs were collected in pre-epidemic influenza season and taken from persons with influenza like illnesses. Virus DNA and RNA were extracted using the NucleoSpin® RNAVirus kit. Multiplex real-time RT-PCR assays performed using Magicplex RV Panel Real-time Test. Real-time PCR performed on the AB 7500 real-time PCR machine.
In this study influenza A, B and pandemic 2009 influenza viruses, respiratory syncytial viruses, human meta-pneumoviruses, adenoviruses, coronaviruses 229E/NL63/OC43, rhinoviruses A/B/C, enteroviruses, bocaviruses and parainfluenza viruses 1, 2, 3 and 4 were identified. Influenza B virus possessed a relatively high frequency, it was identified in 50 % of samples. Human pandemic influenza A(H1N1)pdm 2009, human adenovirus and human bocavirus were found in 25 % of all samples. Human coronavirus 229E/NL63/OC43 observed in 2 samples (12,5 %), and human rhinovirus and human enterovirus found with a low frequency (6,25 %). In more than 40% of cases observed mixed infection, mostly with presence of influenza viruses. To monitor the circulation of respiratory pathogens in humans advisable to use multiplex real-time RT-PCR in pre- and post-epidemic influenza period (May-September). However these studies are not sufficient to answer the question of the contribution of each pathogen of respiratory viral infections in pre-epidemic influenza season.

Keywords: multiplex real-time RT-PCR, respiratory viruses, acute respiratory infections

P60

Host factor preferences of different influenza virus strains

Markus Lesch[1,2], Michael Meyer[2], Isabella Gravenstein[2], Thomas F. Meyer[1], Alexander Karlas[1]

[1]Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany
[2]Steinbeis-Center for Systems Biomedicine, Falkensee, 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, several large loss-of-function screens have been performed by independent laboratories. However, most studies have been performed with the genotypically closely related influenza A virus H1N1 isolates A/Puerto Rico/8/1934 or A/WSN/1933 or genetically modified derivatives of these isolates. Thus, it is unclear if the predetermined host dependency factors (HDF) repertoire is equally important for IV strains with a genotype and phenotype differing from this of the previously employed strains. However, this is important to know in order to design a host-directed anti-IV compound active as broad as possible. To address this issue, we performed an siRNA screen using an array of 1208 recently reported IV HDFs for the replication of four IV strains with differing genotypes and phenotypes (WSN, H3N2, influenza B, H5N1 HPAI). We identified more than 100 factors which were required by all four tested strains. Furthermore, we found several HDFs which were strain- or virus class-specifically required, among others, genes required specifically for the H5N1 HPAI strain or the influenza B strain. Such strain-specific differences were not just found on gene but even on pathway level. Thus, our results indicate that the genotypic differences of IV also translate to the cellular genes and pathways exploited. These findings are highly important for both the development host-directed anti-IV drugs as well as the basic understanding of IV biology.

Keywords: siRNA screen, strain-specific, host factor, H5N1, influenza B

P61

Investigating strain-specific polymorphisms at the influenza A virus NS1-PI3K interface

Antonio P.M.L. Lopes*, Hannah L. Turkington* and Benjamin G. Hale

MRC – University of Glasgow Centre for Virus Research, UK.
*these authors contributed equally

Influenza A virus (IAV) NS1 is a multifunctional virulence factor that binds host p85β and activates cellular phosphoinositide-3-kinase (PI3K). Recent studies suggest a role for naturally-occurring virulence-associated NS1 polymorphisms in modulating PI3K activation[1,2]. However the lack of structure-function analyses on the NS1-p85β interface has so far prohibited rational identification of other polymorphisms impacting this interaction.
Using a structure-based approach, we characterised the 'molecular footprint' of the NS1-p85β interface. Targeted alanine-scanning identified 9 key residues in NS1 specifically required for p85β binding (and not expression, IFN-antagonism or localisation). Notably, 6 of these have not been described previously. Bioinformatic analysis of NS1 sequences derived from >8,000 different IAV strains and hosts identified several strain- and host- specific natural polymorphisms at these 9 important residues, and ongoing studies are focused on investigating their impact with regards PI3K binding. Interestingly, NS1 sequences derived from bat IAVs are highly divergent from non-bat consensus at the p85β interaction site. We have characterised the bat IAV NS1s and found that they are unable to bind human p85β, unlike representative NS1s from other mammalian/avian viruses. Given that the NS1 target site in p85β is (almost) absolutely conserved across avian and mammalian species, including the available sequenced bat species, this suggests that bat IAV NS1s may have evolved to interact preferentially with a novel host/viral factor, or even a divergent p85β specific to a particular host. Overall, our detailed characterisation of the NS1-p85β interface should provide a useful framework for analysing certain natural polymorphisms in the NS1 virulence factor.

References: [1]Li *et al., Vet Res,* 2012 and [2]Fan *et al., J Virol,* 2013

Keywords: NS1, PI3K, polymorphisms, bat influenza, strain-specificity

P62

Influenza B virus induces rapid interferon induction in human macrophages

Sanna M. Mäkelä[1], Pamela Österlund[1], Veera Arilahti[1], Ilkka Julkunen[1,2]

[1]National Institute for Health and Welfare, Virology Unit, Helsinki, Finland. [2]University of Turku, Department of Virology, Turku, Finland.

We have previously shown that influenza B virus (IBV) induces a significantly faster interferon (IFN) response in human dendritic cells compared with influenza A virus (IAV). The early IBV-induced IFN gene expression correlated with the activation of IRF3 transcription factor and took place regardless of virus transcription, replication or protein synthesis. Experiments with UV-irradiated and thus replication incompetent influenza viruses showed that IAV induced IFN responses only after replication of the virus. Now we show that also in human macrophages IBV activates IFN and IRF3 earlier than IAV. To investigate whether IBV triggers the antiviral signaling already from the cell surface, after endocytosis or after the cytosolic release of viral vRNPs, we employed different inhibitors of influenza entry. Dynasore blocks influenza virus entry by inhibiting dynamin-dependent endocytosis needed for clathrin-mediated entry. Bafilomycin A1 inhibits the acidification of the late endosome trapping the virus inside the endosomal compartment by preventing the pH-dependent fusion step of the viral envelope. Both inhibitors reduced IBV-induced early IFN responses and IRF3 activation, which indicates that the IRF3 activation is triggered after the release of viral material into the cytosol. At present it is not known, whether the endocytic routes or the kinetics of IAV and IBV entry are different. However, it seems that IAV is able to evade the early innate immune recognition whereas IBV induces IFN responses directly upon entry.

P63

Identification of amino acid substitutions supporting antigenic change of A(H1N1)pdm09 viruses.

Björn Koel[1], Ramona Mögling[1,2], Salin Chutinimitkul[1], David F. Burke[2,3], Pieter L. Fraaij[1], Emmie de Wit[1], Stefan van der Vliet[1], Theo M. Bestebroer[1], Guus F. Rimmelzwaan[1], Albert D.M.E. Osterhaus[1], Derek J. Smith[2,3], Ron A.M. Fouchier[1], Miranda de Graaf[1,2]

[1] Department of Viroscience, Erasmus MC, Rotterdam, The Netherlands, [2] Center for Pathogen Evolution, Department of Zoology, University of Cambridge, Cambridge, United Kingdom, [3] WHO Collaborating Centre for Modeling Evolution and Control of Emerging Infectious Diseases, University of Cambridge, Cambridge, United Kingdom.

The majority of currently circulating influenza A(H1N1) viruses antigenically resemble the virus that caused the 2009 influenza pandemic. However, antigenic variants are expected to emerge as population immunity increases. Amino acid substitutions in the hemagglutinin protein can result in evasion of neutralizing antibodies, affect viral fitness and change receptor preference. Here we explored amino acid changes in the hemagglutinin that may contribute to appearance of antigenic variants in the human population.
We introduced mutations associated with antigenic changes of influenza A viruses in the hemagglutinin of a representative virus using site-directed mutagenesis.
Antigenic analysis revealed that single substitutions affecting the 152 – 159 loop located adjacent to the receptor binding site caused evasion of ferret and human antibodies elicited after primary A(H1N1)pdm09 virus infection. The majority of these substitutions resulted in similar or increased replication efficiency *in vitro* compared to the virus carrying the wild type hemagglutinin, and did not result in a change of receptor preference. However, none of the substitutions was sufficient to evade the antibodies in sera from individuals that experienced seasonal and pandemic A(H1N1) virus infections.
These results suggest that antibodies directed at epitopes on seasonal A(H1N1) viruses contribute to neutralization of A(H1N1)pdm09 antigenic variants, thereby limiting the number of possible substitutions that could lead to escape from population immunity.

Keywords: Influenza A(H1N1), antigenicity, hemagglutinin, immune evasion, fitness

P64

Shortening the linker region of the non-structural protein NS1 of an avian H1N1 influenza virus increases its replication and pathogenicity in chickens.

Sascha Trapp[1], Denis Soubieux[1], Hélène Marty[1], Pascale Quéré[1], Thibaut Larcher[2], Sandie Munier[3], Nadia Naffakh[3] and Daniel Marc[1]

[1]UMR1282 Infectiologie et Santé Publique, Institut National de la Recherche Agronomique, F-37380 Nouzilly; [2]INRA UMR 703, APEX, Oniris-La Chantrerie, F-44307 Nantes ; [3]Institut Pasteur, Unité de Génétique Moléculaire des Virus à ARN, Département de Virologie, F-75015 Paris

Currently circulating H5N1 viruses all harbor a deletion of amino-acids 80-84 in the unstructured region of NS1 linking its RNA-binding domain to its effector domain. This mutation first appeared in the Hong-Kong region in 2000, and since, H5N1-derived NS segments harboring this mutation have also been found in non-H5N1 viruses. To investigate whether this molecular NS1 signature could alter the viral phenotype, even in non-H5N1 viruses, we genetically engineered this deletion in the NS segment of a duck-origin avian H1N1 virus and compared the *in vivo* and *in vitro* properties of the wild-type and NSdel8084 viruses. The NSdel8084 virus showed both an increased replication potential and an increased pathogenicity in a chicken infection model of influenza. This *in vivo* phenotype was correlated with a higher replicative efficiency *in vitro*, both in embryonated eggs and in a chicken lung epithelial cell line. The increased fitness conferred by this small deletion is therefore a general feature not restricted to H5N1 viruses, suggesting that viruses acquiring this mutation may be positively selected in the future.

Keywords: NS1, linker region, pathogenicity, chicken, replication

P65

Influenza virus non-structural protein NS1 cooperatively binds virus-specific (+)strand RNA sequences

Daniel MARC[1,2], Denis SOUBIEUX[1,2]

[1]UMR1282 Infectiologie et Santé Publique, Institut National de la Recherche Agronomique, Nouzilly, F-37380; [2]UMR1282, Université François Rabelais, Tours, F-37000

Non-structural protein NS1 of influenza viruses is the major interferon-antagonist of influenza viruses, and is also involved in the metabolism of viral and cellular RNAs. Its multiple activities all require a functional RNA-binding domain, the latter being thought to bind non-specifically to several viral and cellular RNAs. We asked whether NS1 could exhibit some sequence-specificity towards its RNA ligands, and performed an *in vitro* selection (SELEX) of NS1-specific aptamers. We identified two virus-specific sequences that are characteristic of the viral RNAs of positive polarity. The first motif, AGCAAAAG, is part of the universal sequence at the 5'-end of all (+)-strand RNAs of influenza A viruses. The second motif, UGAUUGAAG, is highly conserved in NS1-mRNA, 15 nucleotides downstream of NS1's stop codon. In addition, most of NS1-aptamers had one or two symmetrically positioned copies of the 5'-GUAAC / 3'-GUUAC double-stranded motif, which closely resembles the canonical 5'-splice site. We characterized the interaction of NS1 with its RNA-aptamers and showed that NS1's RNA-binding domain specifically recognizes both the sequence and the structure of the virus-specific RNA-sequences. Cooperative binding of NS1's RNA-binding domain leads to its oligomerization on the bound RNA. This strong and intimate interaction suggests that NS1 activity towards viral RNAs is much more specific than previously thought.

Keywords: NS1, RNA binding, specificity

P66

Oncolysis of non-small-cell lung cancer tissue by influenza A virus infection

Masemann, D. [1]; Koether, K. [1,2]; Wixler, V. [1]; Ludwig, S.[1]

[1]Institute of Molecular Virology (IMV), Center for Molecular Biology of Inflammation (ZMBE), University of Münster, Germany; [2]present address: MedImmune Ltd, Cambridge, UK

Lung cancer is the primary cause for cancer-induced deaths worldwide. 85% of all lung cancer diseases belong to the subtype of non-small-cell lung carcinoma (NSCLC). Resistance to radiation and chemotherapy is a typical trait for NSCLC, which results in low cure rates in patients. NSCLC is mainly induced by mutations hyperactivating the Ras-Raf-MEK-ERK MAPK signaling pathway. Based on the facts that: (i) cells of the respiratory tract are the primary targets of influenza A viruses (IAV); (ii) acute IAV infection leads to lysis of target cells and (iii) constitutively active ERK signaling promotes replication of influenza A viruses *in vitro,* we addressed the question whether IAV infection may induce oncolysis of NSCLC *in vivo*.
IAV infection of transgenic mice, developing NSCLC due to the lung-targeted expression of constitutively active human c-Raf-1 (hc-Raf-1-BxB), resulted in a significant decrease of tumor tissue and hc-Raf-1-BxB oncogene expression. Furthermore, tumor foci were primarily infected by the virus and the degree of tumor lysis correlated directly with expression levels of viral RNA. Surprisingly, infected hc-Raf-1-BxB mice exhibited an impaired viral replication and reduced pro-inflammatory cytokine expression compared to WT mice, which was accompanied by enhanced survival of hc-Raf-1-BxB mice after infection. Our data suggest that this might be due to an already pre-activated immune system of uninfected tumor-bearing mice.
In summary, these findings demonstrate an oncolytic effect of IAV infection towards *raf*-transformed lung tumors and indicate the application of IAV as a potential novel approach for therapy of NSCLC.

Note: Parts of these data were presented at different meetings before.

P67

The effect of *Streptococcus suis* co-infection on the infection of well-differentiated porcine respiratory epithelial cells by swine influenza viruses

Fandan Meng[1], Nai-Huei Wu[1], Maren Seitz[2], Peter Valentin-Weigand[2], Xiaofeng Ren[3], Georg Herrler[1]

1. Institute of Virology, University of Veterinary Medicine, Hannover, Germany
2. Institute of Microbiology, University of Veterinary Medicine, Hannover, Germany
3. College of Veterinary Medicine, Northeast Agricultural University, Harbin, China

Disease often occurs due to a combination of various factors including viral and bacterial pathogens as well as environmental factors. A major factor responsible for severe virus infections may be bacterial co-infections. As known, pigs are important hosts for influenza A viruses and may play an important role in the interspecies transmission of influenza viruses. Differentiated airway epithelial cells contain special cell types such as ciliated cells or mucus-producing cells that can't be maintained as immortalized cell cultures. We have recently reported a culture system for differentiated respiratory epithelial cells to analyze the infection of porcine influenza viruses in their natural target cells. Therefore, the aims of this study are to analyze the effect of *Streptococcus suis* (*S.suis*) co-infection on the infection of well-differentiated porcine respiratory epithelial cells by porcine influenza virus types H1N1 and H3N2. Specifically, infection will be analyzed by cells pre-infected with influenza virus. The comparison will reveal to what extent the bacterial infection enhances the severity of infection by porcine influenza virus. We compared five porcine viruses of the three subtypes currently prevalent in the swine populations (H3N2, H1N1, H1N2) with respect to the following parameters: (1) duration of the growth cycle; (2) amount of infectious virus released into the supernatant; (3) extent of the ciliostatic effect. These viruses showed differences in their growth behavior and ciliostatic effect on PCLS and thus reflected the virulence properties of these viruses. Our co-infection studies will reveal whether *S.suis* differentially affects influenza viruses differing in their virulence.

Keywords: respiratory epithelium, precision-cut lung slices, porcine influenza viruses, *Streptococcus suis*

P68

Effects of flavonoid-induced oxidative stress on anti-H5N1 influenza A virus activity exerted by baicalein and biochanin A

Martin Michaelis[1], Patchima Sithisarn[2], Jindrich Cinatl Jr[2]

[1] Centre for Molecular Processing and School of Biosciences, University of Kent, Canterbury CT2 7NJ
[2] Institute for Medical Virology, Clinics of the Goethe-University, Paul Ehrlich-Str. 40, 60596 Frankfurt am Main, Germany

Different flavonoids are known to interfere with influenza A virus replication. Recently, we showed that the structurally similar flavonoids baicalein and biochanin A inhibit highly pathogenic avian H5N1 influenza A virus replication by different mechanisms in A549 lung cells. Here, we investigated the effects of both compounds on H5N1-induced reactive oxygen species (ROS) formation and the role of ROS formation during H5N1 replication. Baicalein and biochanin A enhanced H5N1-induced ROS formation in A549 cells and primary human monocyte-derived macrophages. Suppression of ROS formation induced by baicalein and biochanin A using the antioxidant N-acetyl-L-cysteine strongly increased the anti-H5N1 activity of both compounds in A549 cells but not in macrophages.These findings emphasise that flavonoids induce complex pharmacological actions some of which may interfere with H5N1 replication while others may support H5N1 replication. A more detailed understanding of these actions and the underlying structure-activity relationships is needed to design agents with optimised anti-H5N1 activity.

Keywords: H5N1, antiviral, reactive oxygen species, N-acetyl-L-cysteine

P69

Investigation of influenza A virus M and NS segments splicing control

Olivier Moncorgé [1], Elliott Jennings [1], Wendy S Barclay [1].

1 Section of Virology, Department of Medicine, Imperial College London, St. Mary's Campus, London, United Kingdom

Influenza A virus segments M and NS produce spliced and unspliced viral mRNAs. Regulation of influenza splicing is not well understood. It is believed that both virus and cellular factors are involved in the kinetics of viral mRNA splicing, and inefficient splicing control has been suggested to be responsible for abortive replication in some models. Since influenza mRNAs are distinct from cellular mRNA, for example being transcribed by the viral polymerase rather than RNA polymerase II, splicing regulation might be different from that of most host cell messages.
We have developed a luciferase reporter assay to measure expression of the three mRNAs products from the M gene: M1 (unspliced) and M2 and m3 (spliced), and the two NS gene products: NS1 (unspliced) and NS2/NEP (spliced). The assay is a modification of the *in situ* polymerase assay in which a functional viral polymerase is reconstituted in the cell nucleus. We confirmed that the viral protein NS1 modulates M and NS genes pre-mRNA splicing. We tested whether the nature of the viral polymerase that directs viral mRNA transcription affects splicing efficiency. Investigating a role for the host cell in influenza splicing control, we compared splicing in cell types of mammalian and avian origins. We have discovered that splicing efficiency varies in human cells depending on the origin of the viral polymerase used. This may explain the host range barrier that means most avian influenza viruses do not readily infect humans.

Keywords: splicing, polymerase, host range, adaptation

P70

Altered Growth Kinetic and Cell Tropism for the 2009 Pandemic H1N1 by Single Reassortment of the NS Segment

Ahmed Mostafa[1,2], Henning Petersen[3], Balachandar Selvakumar[4], Susanne Herold[4], Silke Rautenschlein[3] and Stephan Pleschka[1]

[1]Institute of Medical Virology, Justus-Liebig University-Giessen, BFS, Schubertstrasse 81, Giessen, Germany
[2]Virology Laboratory, Environmental Research Division, National Research Center, 12311 Dokki, Giza, Egypt
[3]Clinic for Poultry, University of Veterinary Medicine Hannover, Buenteweg 17, D-30559 Hannover, Germany
[4]Department of Internal Medicine II, University of Giessen Lung Center (UGLC) and German Center for Lung Research (DZL), Giessen, Germany

Since the appearance and wide spread of the pandemic H1N1 influenza A virus (H1N1pdm09) in 2009, there is a main concern about the possibility of reassortment between such viruses and circulating highly pathogenic avian influenza viruses (HPAIVs). This concern is in part derived from the fact that H1N1pdm09 can infect turkey and possibly other bird species and could therefore recombine with avian influenza viruses and might have unexpected and unknown implications for animal and human health. By reverse genetics we explored the effect of NS segment reassortment between the H1N1pdm09 strain A/Giessen/06/09 (Gi-H1N1) and other human H1N1 influenza A virus, HPAIV of H5- and H7-subtypes and low pathogenic avian influenza virus (LPAIV) of the H7- and H9-subtype on the viral characteristics. We noticed a significant promotion in growth kinetic and change in host cell tropism of reassortant virus with NS segments of A/FPV/Rostock/34 (H7N1) and MDCK-adapted reassortant strain harboring NS segment of A/Puerto Rico/8/34 (H1N1) in secondary human, avian and porcine cell culture and in primary avian tracheal organ cultures. The Gi-H1N1 reassortants with NS segments of A/Wilson Smith N/33 (WSN; H1N1), A/Goose/Guangdong/1/96 (GD; H5N1), A/Thailand/KAN-1/2004 (KAN-1; H5N1), A/Mallard/NL/12/2000 (Ma; H7N3), A/FPV/Bratislava/79 (FPV-B; H7N7), A/Anhui/1/2013 (Anhui; H7N9) and A/chicken/Saudi Arabia/CP7/98 (SA; H9N2) showed replication efficiencies comparable to the parental H1N1-Gi. While, the NS segment of A/Victoria/3/75 (Victoria; H3N2) reduced the replication efficiency of the Gi-H1N1 in different secondary cell lines. These results confirm that possible reassortment of H1N1pdm09 with different influenza A viruses should be continuously monitored and furthermore investigated *in vitro* and *in vivo* to predict the implications of such reassortant viruses for animal and human health.

Keywords: NS-reassortant, pandemic H1N1, Non-structural protein (NS)

P71

Cloning Strategies for Unstable Influenza Hemagglutinin Segments: Towards Rapid *In Vitro* Vaccine Production

Ahmed Mostafa[1,2#], Pumaree Kanrai[2], Henning Petersen[3], Sherif Ibrahim[4], Silke Rautenschlein[3], John Ziebuhr[2] and Stephan Pleschka[2*]

[1]Center of Scientific Excellence for Influenza Viruses, National Research Center (NRC), Dokki, Giza, Egypt
[2]Institute of Medical Virology, Justus Liebig University Giessen, Schubertstrasse 81, 35392 Giessen, Germany.
[3]Clinic for Poultry, University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany.
[4]Department of genetic engineering, Veterinary Serum and Vaccines Research Institute (VSVRI), Agricultural Research Center (ARC), El-Seka El-Beda street, Abbassia 131, Cairo, Egypt.

Influenza A viruses (IAVs) are the most relevant and continual source of sever infectious respiratory complications in humans and poultry. Therefore, an efficient vaccination that elucidates protective and neutralizing antibodies against the viral hemagglutinin (HA) and neuraminidase (NA) is an important strategy to face and control annual epidemics or occasional pandemics caused by emerging IAVs. With the help of plasmid-based reverse genetics technology, it is possible that candidate vaccine seed virus (CVSV) are rapidly generated by combination of newly cloned HA and NA cDNAs from IAV of concern with the plasmids encoding the other viral segments of a high-yield donor virus to prepare either inactivated or live attenuated vaccines within few weeks after the isolation of the circulating epidemic or pandemic IAV. However, the correct and high quality production of the plasmids encoding the cDNAs corresponding to the HA and NA segments in bacterial cells is an essential step for the successful and efficient rescue of the CVSV by recombinant DNA technology. The instability of some HA-cDNAs cloned and transformed into competent bacteria represents a major obstacle. Here we offer a solution for this problem as demonstrated by efficient cloning of different unstable HA segments (H5- and H9-subtypes) employing a newly constructed vector for reverse genetics (pMKP*ccd*B) and an old *Escherichia coli* strain.

Keywords: IAV, Hemagglutinin instability, pMKP*ccd*B, *Escherichia coli*

P72

Ferret glycomics reveals different sialylated glycans to humans: Implications for their use in Influenza Virus Infection Studies

Nan Jia[1], Wendy S. Barclay[2] . Hui-Ling Yen[3], Renee W,Y Chan[3], Alfred KY Lam[4], Gillian Air[5], JS Malik Peiris[3], Anne Dell[1], Stuart M. Haslam[1]* and John M Nicholls[6]*

[1]Department of Life Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom, Imperial College London, [2]Faculty of Medicine, Division of Infectious Disease, Norfolk Place, London, W2 1PG, United Kingdom, School of Public Health[3], The University of Hong Kong, Pokfulam, Hong Kong SAR, China, [4]Department of Pathology, Griffith University, Queensland, Australia, [5]Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, PO Box 26901,Oklahoma City, OK 73126-0901 and [5] Department of Pathology, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

Influenza binds to sialylated glycans in the respiratory tract. We have previously published data on the human and pig glycans present in the upper and lower respiratory tract. The ferret has been extensively used as an animal model to study the transmission of influenza viruses as it shares many clinical and physiological features with human influenza infections. To better understand the validity of this animal model system we undertook glycomic characterization of the upper and lower respiratory tract tissues of ferret which allows a comparison of potential viral receptors to be made between human and ferret. To complement the structural analysis, lectin binding and sialyltransferase expression experiments were carried out to characterize the regional distributions of glycans along the respiratory tract of the ferret. Lastly, the binding affinities between glycans identified and hemagglutinins of different strains of influenza viruses were assessed by glycan array experiments. Our data indicated that the respiratory tissues of ferret heterogeneously express both α2-3 and α2-6 linked sialic acids, with the latter being more abundant. However, the respiratory tissues of ferrets, in particular the trachea, bronchus and nasal mucosa also expressed the Sda epitope (NeuAcα2-3(GalNAcβ1-4)Galβ1-4GlcNAc) and sialylated LacdiNAc (NeuAcα2-6GalNAcβ1-4GlcNAc), which have not been observed in the human respiratory tract surface epithelium. The presence of this Sda epitope reduced potential binding sites for avian viruses and the lectins MAA-I and MAA-II, and thus may have implications for the usefulness of the ferret in the study of influenza virus infection and its correlation to human infection studies.

P73

Tetrameric and dimeric MxA proteins recognise influenza A virus nucleoprotein that is not incorporated in vRNPs after 1° transcription

Patricia E. Nigg[1], Dominik Mueller[1], Linda Brunotte[2], Dominik Dornfeld[2], Michel Crameri[1], Martin Schwemmle[2] and Jovan Pavlovic[1]

[1] Institute of Medical Virology, University of Zürich
[2] Institute for Virology, University Medical Centre of Freiburg

The interferon-induced human MxA protein exerts a broad antiviral activity against negative stranded RNA viruses including influenza A (IAV). The recent atomic resolution of the MxA structure revealed that it can form stable tetramers and oligomers. However, whether these tetramers of MxA or higher ordered oligomeric structures represent the antiviral entity and to bind to components of the viral ribonucleoprotein (vRNP) remains to be shown. Biochemically characterised MxA mutants with a defined oligomerisation state revealed that the tetrameric, dimeric and the monomeric form of MxA exhibited antiviral activity. Since previous studies have shown that the nucleoprotein (NP) of IAV plays an important role in determining sensitivity or resistance to MxA, we also tested whether these MxA mutants interact with NP by transient transfection and co-immunoprecipitation. Intriguingly, only the tetrameric and dimeric forms of MxA were able to bind NP efficiently. To determine whether MxA associates with vRNPs, MxA expressing cells were infected with a recombinant virus expressing a strep-tagged PB2, allowing the purification of vRNPs. Enrichment of vRNPs failed to co-purify detectable amounts of MxA. These results suggest that the antiviral active tetrameric and dimeric form of MxA preferentially recognise free NP. According to these results we suggested a model where MxA captures free NP which consequently leads to the inhibition of IAV replication. To confirm this theory we infected cells pre-treated with cycloheximide. RT qPCR clearly shows that MxA inhibits IAV replication after 1° transcription, supporting our model of MxA preventing IAV replication by capturing newly synthesised NP.

P74

A new aspect of Influenza virus pathogenesis

A.A.Azarenok[1], A.R.Prochukhanova[1], E.V.Ilynskaya[1], V.V. Zenin [2],O.G. Lublinskaya [2], E.M.Eropkina[1], I.N.Zhilinskaya[1].

[1]Research Institute of Influenza, St. Petersburg, Russia; [2]Institute of Cytology RAS, St. Petersburg, Russia

BACKGROUND: The aim of the study was to investigate the role of endothelium in the pathogenesis of influenza infection.

OBJECTIVES AND METHODS: We have used the culture of human endothelial cells EAhy926; influenza virus type A subtypes H1N1pdm 09, H3N2, H5N1; HA and NA surface proteins of influenza A virus.

RESULTS: We found that influenza virus type A (subtypes H5N1, H3N2, H1N1 pdm 09) is capable of:
1. reproducing in EAhy926 cell culture (3,5-4,16 lg TCD_{50}/ml). These data are confirmed by detection of HA and NP in endothelium of lung blood vessels during analysis of autopsy material from patients who died from influenza during 2009-2010 epidemic.
2. inhibiting intracellular dehydrogenases (20-40%) and causing apoptosis of endothelial cells (an increase in the amount of early apoptotic cells by 6.8% compared with control).
3. causing 2-3 times increase in activity of tissue plasminogen activator (t-PA) in human endothelial cells in vitro compared with the control.

HA and NA also cause inhibition of intracellular dehydrogenases (20% and 40%, respectively), an increase in the activity of t-PA in vitro (2-3 times compared to the control) and in vivo (4-5 times). NA stimulates the appearance of early apoptotic cells (6% to the control)

CONCLUSIONS: Influenza A virus is capable of reproducing in endothelial cells and causing their dysfunction.

P75

Influenza A virus suppresses cyclooxygenase-2 expression by affecting mRNA stability

Katja Nitzsche, Rüdiger Dierkes, Lena-Maria Makowski, Stephan Ludwig and Christina Ehrhardt

Institute of Molecular Virology, Centre of Molecular Biology of Inflammation, University of Muenster, Von- Esmarch- Straße 56 48149 Muenster, Germany

Influenza A viruses (IAV) are the causative agents for the acute respiratory disease influenza that occurs in seasonal outbreaks accompanied with fever, coryza, cough or malaise. In addition to those annual epidemics, IAV also possess a frightening pandemic potential with enormous morbidity, mortality and economic losses.
In humans IAV primary infect epithelial cells of the upper respiratory tract and the trachea. Upon IAV infection the innate immune response is the first unspecific defence of the host, resulting in production and release of type I interferon, pro-inflammatory cytokines, chemokines and mediators as part of the inflammatory response. Cyclooxygenases (COX) are important autocrine and paracrine mediators that catalyse the synthesis of prostanoids derived from arachidonic acids. The isozyme COX-2 is responsible for the formation of prostaglandins which are involved in the inflammatory response.
Despite the fact that IAV-regulated induction of COX-2 has been proven, we demonstrate a significant down-regulation during IAV infection in human lung epithelial cells (A549). COX-2 protein expression initially increases upon recognition of viral RNA by the pathogen recognition receptor RIG-I. Nonetheless, at on-going IAV replication we recognised the suppression of the COX-2 protein and mRNA expression attended with a considerable loss of mRNA stability. Furthermore, our results revealed a virus-dependent regulation of COX-2 executed by tristetraprolin (TTP), which is known to bind COX-2 mRNA and subsequently promotes its rapid degradation. Thus, we identified an additional mechanism of IAV to evade host defence that supports viral replication.

Keywords: Influenza A viruses, Cyclooxygenase-2, RIG-I signalling, mRNA stability, Tristetraprolin

Note: Parts of these data were presented at different meetings before.

P76

Promotion of secretory protein synthesis by the NS1 RNA-binding domain is regulated by its effector domain

Johan Nordholm

Stockholm University, Department Biochemistry & Biophysics

Influenza NS1 is a two-domain protein that suppresses the immune response during infection by binding to key host proteins through interactions with its RNA-binding domain (RBD) and effector domain. Here we show that of the 10 major influenza proteins, NS1 predominantly enhances the synthesis of the secretory proteins NA and HA. The enhancement is driven by the RBD, is conserved between phylogenetically diverse NS1s, and appears to be general for membrane glycoproteins. The function of the RBD requires its RNA-binding activity and is regulated by its effector domain as only a few natural substitutions in this domain could dramatically reduce or slightly increase glycoprotein expression, compared to the RBD alone. Localization analysis indicates that this function of the RBD correlates with its presence in discrete nuclear domains. This data shows that in addition to its role in immune evasion, NS1 plays a key role in balancing protein levels to ensure an optimal replication and is likely involved in the reprogramming of cell synthesis.

P77

Virus and host determinants of influenza A virus restriction by human MxA

Corinna Patzina, David Riegger, Georg Kochs

Institute of Virology, University Medical Center Freiburg, Germany

Human myxovirus resistance protein (MxA) is an interferon-induced effector with a broad spectrum of antiviral activity against divergent viruses, e.g. orthomyxo- or bunyaviruses. MxA-mediated inhibition of influenza A virus (FLUAV) replication is dependent on the virus strain, as viruses of avian origin are more sensitive to MxA than human strains. An accessible cluster of amino acids in the viral nucleoprotein (NP) determines MxA sensitivity, implying that MxA specifically recognizes FLUAV NP. However, it is still unclear how MxA can inhibit the replication of such a broad range of viruses.
To identify sites in human MxA that determine its specificity to FLUAV and to related orthomyxoviruses, a comparative analysis of primate MxA proteins was performed, revealing the overall high conservation of MxA, with a limited set of amino acids evolving under strong positive selection, a typical signature of host-pathogen interfaces. These residues mainly cluster in loop L4, a surface-exposed, flexible domain that protrudes from the highly ordered stalk of MxA. We further characterized the minimal motif in loop L4 of MxA required for orthomyxovirus specificity and vRNP association, and were able to show that most of these residues are not critical for bunyavirus recognition.
We thus hypothesize that loop L4 is a flexible and modular structure that evolved as recognition interface for a broad range of viral pathogens and that MxA specifically restricts FLUAV by a direct interaction of loop L4 with NP, most likely by multiple contacts of MxA oligomers with FLUAV nucleocapsids.

Keywords: Innate immunity, MxA protein, influenza A virus, orthomyxoviruses

P78

Pulmonary immunization of chickens using non-adjuvanted spray-freeze dried whole inactivated virus vaccine completely protects against highly pathogenic H5N1 avian influenza virus.

Ben Peeters[1], Wouter F. Tonnis[4], Senthil Murugappan[4], Peter Rottier[2], Guus Koch[1], Henderik W. Frijlink[4], Anke Huckriede[3], Wouter L.J. Hinrichs[4]

[1] Central Veterinary Institute of Wageningen University and Research Centre, Lelystad, The Netherlands; [2]Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands; [3] Department of Medical Microbiology, University Medical Center Groningen, The Netherlands; [4] Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, The Netherlands

Highly pathogenic avian influenza (HPAI) H5N1 virus is a major threat to public health as well as to the global poultry industry. Most fatal human infections are caused by contact with infected poultry. Therefore, preventing the virus from entering the poultry population is a priority. To evaluate the suitability of needle-free pulmonary immunization for mass vaccination of poultry against HPAI H5N1, we performed a proof-of-concept study in which we investigated whether non-adjuvanted spray-freeze-dried (SFD) whole inactivated virus (WIV) can be used as a dry powder aerosol vaccine to immunize chickens. Our results show that chickens that received SFD-WIV vaccine as aerosolized powder directly at the syrinx (the site of the tracheal bifurcation) mounted a protective antibody response after two vaccinations and survived a lethal challenge with HPAI H5N1. Furthermore, both the number of animals that shed challenge virus, as well as the level of virus shedding, were significantly reduced. Based on antibody levels and reduction of virus shedding, pulmonary vaccination with non-adjuvanted vaccine was at least as efficient as intratracheal vaccination using live virus. Animals that received aerosolized SFD-WIV vaccine by temporary passive inhalation showed partial protection (22% survival) and a delay in time-to-death, thereby demonstrating the feasibility of the method, but indicating that the efficiency of vaccination by passive inhalation needs further improvement. Altogether our results provide a proof-of-concept that pulmonary vaccination using an SFD-WIV powder vaccine is able to protect chickens from lethal HPAI challenge and may be suitable for mass application.

Keywords: avian flu, powder vaccine, inhalation, poultry vaccine, mass vaccination

P79

Small RNA deep sequencing reveals host strain-dependent differences in pulmonary microRNA expression in influenza A virus infected mice

Matthias Preusse[1,4], Mohamed Samir[1,2], Mahmoud BahgatRiad[1], Klaus Schughart[3], Frank Pessler[1,4] (corresponding author)

[1]Institute for Experimental Infection Research, TWINCORE Center for Experimental and Clinical Infection Research, Feodor-Lynen-Str. 7, 30625 Hannover, Germany
[2]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
[3]Dept. of Infection Genetics, Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany
[4]Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany

Background: Expression of several host encoded microRNAs is known to be regulated during influenza A virus (IAV) infection in normal and adaptive hosts and may play important roles in regulating both susceptibility and resistance to IAV infection. However it is unknown whether miRNA expression can be linked directly to genetically determined host susceptibility to IAV infection. We therefore compared changes in pulmonary miRNA expression during IAV infection in two inbred mouse strains with greatly differing susceptibility to IAV.
Methods and Results: Using small RNA sequencing, miRNA expression profiles were determined in lungs of the relatively susceptible mouse strain DBA/2J and the relatively resistant strain C57BL/6J at 6, 12, 18, 24, 48, and 120 h post infection (hpi) with mouse adapted IAV PR8M. Even the miRNomes of uninfected lungs differed substantially between the two strains. After a period of relative quiescence, major reprogramming was detected in both strains at 48 hpi and increased through 120 h. Fold change of expression was usually higher in the DBA/2J strain. Application of successive filters identified several miRNAs whose expression correlated with HA mRNA expression but differed between the two host strains. Network analysis revelealed significant regulation of miRNAs associated with regulating apoptosis and the Pi3K pathway.
Conclusions: This study suggests that host strain-dependent differences in abundance and regulation of host miRNas likely contribute to murine genetic susceptibility to IAV infection. It also underscores the importance of apoptosis and the PI3K pathway in early IAV infection.

P80

UBR box N-recognin-4 (UBR4) is a human host factor required by influenza A virus during late stages of the viral life cycle.

Pohl MO[1*], Tripathi S[2*], König R[3*], Zhou Y[7], De Jesus PD[4], Moulton HM[5], Stein DA[5], Shaw ML[2], Krogan NL[6], García-Sastre A[2#], Chanda SK[4#], Stertz S[1#]

[1] Institute of Medical Virology, University of Zurich, Zurich, Switzerland
[2] Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA
[3] Division of Medical Biotechnology, Paul-Ehrlich-Institute, Langen, Germany
[4] Infectious and Inflammatory Disease Center, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA
[5] Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR, USA
[6] Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA, USA
[7] Genomics Institute of the Novartis Research Foundation
*,# these authors contributed equally

The identification of human host factors that favor viral propagation can promote the development of novel antiviral drugs against influenza A virus (IAV). Recently, genome-wide siRNA screens have been performed to determine such factors. Surprisingly, there was only little overlap between the primary hits identified in the individual screens. A meta-analysis of the raw RNAi screen data identified, among others, UBR box N-recognin-4 (UBR4), a member of the N-recognin family, as a host factor required by IAV. Moreover, a proteomic screen for interactors of viral proteins revealed UBR4 as interaction partner of the viral ion channel M2.
Our follow-up studies showed that siRNA-mediated knockdown of UBR4 reduced infectious particle release during both, mono- and multicycle growth. However, viral NP production as well as replication of a single cycle reporter virus remained unaffected by UBR4 knockdown. These data indicate that UBR4 is required during a late step of the viral life cycle. Importantly, knockdown of UBR4 in mice using peptide-phosphorodiamidate morpholino oligonucleotides (PPMOs) resulted in significant reduction of viral titers in the lung and thus shows that UBR4 is also required for IAV propagation *in vivo*. Ongoing studies aim to elucidate the molecular mechanism of UBR4 function during late steps of viral replication.

P81

Adaptation of pandemic H1N1 in different mice models

Prokopeva E.A.[1,2,*],Glushenko A.V.[1], Shestopalov A.M.[1,2]

1 Research Center of Clinical and Experimental Medicine Siberian Division of the Russian Academy of Sciences
2 Novosibirsk State University
*presenting author

We have conducted a study on the adaptation of pandemic H1N1 by serial sequential lung-to-lung passages in mice BALB/c (n = 145), C57Bl/6z (n = 245). The adapted strains caused 100% mortality in experimental animals. Stamm A/Tomsk/273-Balb_C/2010-MA(H1N1) was derived after total 7 sequential lung-to-lung passage in mice BALB/c, and stamm A/Tomsk/2733-C57Bl_6z/2010-MA(H1N1) was derived after total 12 sequential lung-to-lung passaged in mice C57Bl/6z. The cross-infection have been conducted in order to identify characteristics of the course of influenza infection in a clearly differentiated from each other genetic groups of mouse. The experimental results showed that the mouse-adapted strain on mice BALB/c is highly virulent for mice C57Bl/6z, causing 100% mortality, whereas the mouse-adapted strain C57Bl/6z is not lethal to mice BALB/c. The morphological analysis showed different damages in both groups of infected animals: in the lung tissue revealed the development of pneumonia, multiple hemorrhages, infiltration, reduced airiness of the lung tissue; in brain tissue account of the perivascular edema, multiply hemorrhage, vascular congestion; in the liver, kidney and in the heart are also observed degenerative changes. For each mouse-adapted variants were produced and were subjected to sequence analysis to determine whether they shared common mutations. Besides, it analyzes the current data on the role of the individual segments of the genome to increase the virulence of the influenza virus in laboratory adaptation in the lung tissue of mice.

Keywords: adaptation, H1N1, mice BALB/c and C57Bl/6z

P82

Circumvention of importin-α7 requirement is mediated by adaptive mutations in the viral RNP complex and the viral surface glycoproteins.

Patricia Resa-Infante [1], Duncan Paterson [2], Jaume Bonet [3], Anna Otte [1], Baldo Oliva [3], Ervin Fodor [2] and Gülsah Gabriel [1]

[1] Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany; [2] Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom; [3] Structural Bioinformatics Lab (GRIB-IMIM), Universitat Pompeu Fabra, Barcelona Research Park of Biomedicine (PRBB), Barcelona, Spain

Importin-α7 (α7), a component of the cellular nuclear import pathway, is crucial for mammalian influenza virus replication and pathogenicity. Mice with a deleted importin-α7 gene ($\alpha7^{-/-}$) survive infection with a 2009 pandemic H1N1 (pH1N1) influenza virus which causes 100% lethality in wildtype (WT) mice. This raises the possibility that α7 could be used as a new antiviral target to combat human influenza. In this study, we addressed the question whether influenza viruses could circumvent the requirement for α7. Therefore, we have adapted a pH1N1 isolate by serial lung-to-lung passaging in $\alpha7^{-/-}$ mice. Only 5 passages were required to reach 100% lethality in $\alpha7^{-/-}$ mice. Sequencing of a $\alpha7^{-/-}$ mice-adapted viral pH1N1 clone (named pH1N1-MA7) revealed 5 adaptive mutations located in the viral ribonucleoprotein (RNP) complex (PA-D27E and NP-G102R) and the viral surface glycoproteins (HA-K171E, HA-D239G and NA-Y170H). Interestingly, the adapted pH1N1-MA7 virus strain was not only more pathogenic in $\alpha7^{-/-}$ but also in WT mice. However, only the adaptive mutations in the RNP specifically elevated pathogenicity in $\alpha7^{-/-}$ mice. Moreover, the increased virus replication displayed by pH1N1-MA7 was still partially dependent on the expression of α7. These results show that circumvention of the requirement for α7 in mice is mediated by adaptive mutations in the RNP complex as well as in HA and NA. Overall, these studies may underpin antiviral strategies in which both host and viral proteins could be targeted in order to reduce the risk of viral escape.

Keywords: Pathogenesis, virus evolution, host adaptation

P83

Stable introduction of a variety of fluorescent and luminescent reporter genes as well as a Cre-recombinase gene into the NS-segment of Influenza A viruses.

Peter Reuther, Kristina Göpfert, Alexandra Dudek, Dominik Dornfeld, Martin Schwemmle

Institute for Virology, University of Freiburg, 79104 Freiburg, Germany

Reporter viruses represent valuable tools to study the dynamics of a viral infection in cell culture and animal models. Our study presents a novel strategy allowing the stable expression of a variety of genes in influenza-infected cells without fusion of the corresponding proteins to viral factors. This is accomplished by the insertion of the desired gene between the separated ORFs for NS1 and NEP within the NS segment. The respective gene products are co-translationally separated by two genetically modified PTV-2a peptides. Using this method, we generated A/SC35M (H7N7) viruses encoding Renilla luciferase, secreted Gaussia luciferase, GFP-derivates (GFP, dsRed, Azurite) as well as a virus encoding a Cre-recombinase.
To evaluate the suitability of the luciferase-encoding viruses for drug screenings, we treated infected MDCKII cells with the nucleosid inhibitor ribavirin. Especially the Gaussia-encoding virus proved to be a valuable tool for this purpose, since luciferase activity can be measured in the supernatant and does not require cell lysis.
The Cre/loxP system is a widely used method to control the expression of target genes. To employ this method for influenza virus research, we generated a virus encoding a Cre-recombinase. To prove that we can utilize it as a tool to regulate gene expression specifically in influenza infected cells, we infected human Calu-3 cells transduced with a loxP_dsRed_loxP_GFP construct. Indeed, infected cells switched from red to green fluorescence, and in vivo studies using suitable reporter mice are ongoing. Intriguingly, this method allows for the first time the identification and characterization of cells that can survive an influenza virus infection.

Keywords: Reporter viruses, drug screening, Cre-recombinase

P84

Escape from Mx restriction: an H7N7 avian influenza virus with enhanced virulence in interferon-competent mice

Christoph Deeg, Pascal Mutz, Lara Rheinemann, Carsten Kallfass, Cindy Nürnberger, Mirjam Schilling, Sébastien Soubies & Peter Staeheli

Institute for Virology, University Medical Center Freiburg, Freiburg, Germany

In mice, influenza virus resistance is strongly dependent on the interferon (IFN)-regulated *Mx1* gene which encodes a potent restriction factor that inhibits a poorly defined early step of the viral life cycle. Humans possess two IFN-regulated *Mx* genes with antiviral activity. The human *Mx1* gene (encoding MxA protein) confers resistance to a broad range of RNA viruses in cell culture, including influenza A viruses, whereas the human *Mx2* gene (encoding MxB protein) has been shown to inhibit HIV-1.
To determine whether human MxA plays a decisive role in defending the intact organism against influenza A viruses, we developed a mouse strain that lacks functional endogenous *Mx* genes but carries the complete human *Mx* locus as a transgene. MxA-transgenic mice exhibited solid resistance to infection with highly pathogenic H5N1 and H7N7 avian influenza viruses as well as influenza-like Thogoto virus. However, transgenic mice differed only slightly from non-transgenic littermates with regard to resistance to H1N1 and H3N2 influenza viruses of human origin, suggesting that seasonal human influenza viruses have acquired adaptive mutations which permit MxA evasion.
To identify adaptive mutations which confer Mx resistance, we passaged mouse-adapted H7N7 avian influenza virus SC35M in MxA-transgenic mice. Here we will describe an escape variant of SC35M resulting from this screen that induces fatal disease in MxA-transgenic mice and exhibits enhanced virulence for mice with intact endogenous *Mx1* genes.

Keywords: interferon, Mx, avian influenza virus, adaptation

P85

Mass-spectrometry based profiling of PKR-interaction partners in the course of influenza A virus infection

S. Sänger[1], R. Daviña Nuñez [1], T. Wolff[1]

[1]Robert Koch-Institute, Division 17, Influenza Viruses and Other Respiratory Viruses, Berlin, Germany

PKR is an interferon induced, double-stranded RNA-activated protein kinase, which plays a significant role in innate immunity. The activation of PKR leads to dimerization, autophosphorylation and phosphorylation of numerous downstream factors. Downstream effects of PKR activation include inhibition of translation, initiation of apoptosis and the induction of transcription factors that lead to production of type I interferon. Due to its key role in antiviral immunity many viruses have evolved mechanisms to avoid PKR activation. We and others have previously described the influenza virus non-structural protein 1 (NS1) as a protein antagonist of PKR. We are interested in understanding the precise mechanism of PKR activation in the context of influenza virus infection and the role of cellular and viral factors in regulating PKR activation. For this purpose we used Stable-Isotope-Labeling-by Amino-acids-in-Cell-culture (SILAC) followed by LC-MS/MS analysis to identify immunoprecipitable interaction partners of PKR before and during influenza wildtype or delNS1 virus infection. In four replicates we were able to detect more than 180 cellular PKR binding partners. Among these 54 interactors were only found in wildtype infected cells whereas 29 were characteristic for delNS1 virus infection. Bioinformatic analysis indicates that the majority of these particular proteins are involved in cellular pathways as RNA processing, stress response and apoptosis. We are in the process of validating a subset of the detected proteins. It is expected that bioinformatic and functional analyses of novel PKR interaction partners will further our understanding of cellular antiviral mechanisms and their modulation by influenza A virus.

Keywords: PKR, Influenza, innate immunity, Mass-spectrometry

P86

Kinetics of viral spread and histopathological changes in ducks experimentally infected with the highly pathogenic avian influenza A virus strain A/duck/Zagazig/amn13/2011 (H5N1)

Mohamed Samir[1,3,4], Ashraf Hamed Hussein[4], Fatma Ahmed Abdulla[4], Ahmed Abd El-Samie Hassan Ali[4], Frank Pessler[1,2]

[1]TWINCORE, Center For Experimental and Clinical Infection Research, Hannover, Germany
[2]Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]University of Veterinary Medicine, Hannover, Germany
[4]Faculty of Veterinary Medicine, Zagazig University, Egypt

Highly pathogenic avian influenza (HPAI) viruses usually cause asymptomatic infection in ducks, however, recent isolates were proved to be lethal in this species. In this study, 2-week-old Balady ducks were intranasally inoculated with 10^5 EID_{50} HPAI A/duck/zagazig/amn13/2011 (H5N1, clade 2.2.1), which was isolated in Egypt from commercial ducks died from fulminant infection. Clinical signs and post mortem lesions were recorded at 8, 16, 24, 32, 40, 48, 56, and 72 hours post infection (h.p.i). Virus titer, immunohistochemical expression of viral NP, and severity of histopathological changes using a newly devised histopathological scoring system were determined. At 40 h.p.i., the infected ducks showed fever, depression and anorexia. Congestion in some organs was seen upon necropsy. At 56 h.p.i, ducks died after exhibiting sever nervous manifestations, including torticolosis and blindness. In general, virus titer in lung and trachea was higher than in heart, brain and pancreas. In most of the organs, viral replication peaked at 48 h.p.i., except for lung where replication persisted until 56 h.p.i. In heart and trachea, virus titer dropped slowly after 48 h.p.i., but in pancreas, it declined sharply by this time. Pancreas and heart showed the most sever histological lesions. In brain and trachea, histopathological lesions were more widespread than NP antigen staining. Pancreatitis, pericarditis, mild tracheitis and mild encephalitis were the most frequently observed histopathological lesions. Our experimental results demonstrate the systemic spread of this HPAI virus and that virus replication in the heart and the brain might account for the death of the infected duck.

Keywords: ducks, H5N1, histopathology, influenza A virus

P87

In vitro and in vivo evaluation of the inhibitory potential of azadirachta indica juss (neem) leaf extract against influenza a virus replication

Latika Saxena*, Roopali Rajput, Dibya Ranjan Pati, Anju Gautam, Madhu Khanna

Department of Respiratory Virology, VP Chest Institute, University of Delhi, Delhi-110007

Influenza A viruses are known to cause widespread epidemics and pandemics with high mortality owing to their high rate of mutation. Rapid emergence of drug resistance against the currently available antiviral drugs and the limited use of influenza vaccines have exposed the need for the development of alternative antiviral strategies against influenza. In this study, Methanol extract of *Azadirachta indica* leaves was prepared and tested for its cytotoxic activity by colorimetric assay. The antiviral activity was evaluated at different concentrations of the extract against A/Udorn/307/72 (H3N2) infected MDCK cells by cytopathic effect (CPE), hemagglutination assay (HA), real time RT-PCR and western blotting. The potent anti influenza effect of the extract was evident by the dose dependant reduction in CPE of MDCK cells and decrease in the HA titer. Real time RT-PCR showed 40% inhibition in the expression of HA gene of the virus. The same was observed by western blot analysis. Time response study revealed that the antiviral activity of the extract was highest at 2-6 h post infection. The extract also exhibited antiviral potential in vivo in BALB/c mice which was shown by histopathologic examination of the mice lung and evaluation of virus titer in the lung homogenate. The results reflect that the leaf extract of *Azadirachta indica* is inhibits the growth of influenza A virus and can be used in future for the development of an alternative and effective antiviral therapy against influenza A viruses.

P88

Role of NS1 phosphorylation for influenza A virus replication in human lung epithelial cells

T. Schräder[1], C. Nordhoff[1], D. Anhlan[1], V. Wixler[1], and S. Ludwig[1]

[1]Institute of Molecular Virology (IMV), Centre for Molecular Biology of Inflammation (ZMBE), Westfaelische Wilhelms-Universitaet Muenster, Germany

Influenza A virus (IAV) has evolved several strategies to counteract host immune responses. The non-structural protein 1 (NS1) of IAV was identified as a major interferon antagonist of the virus. It dampens host immune responses and enhances viral replication by interfering with host cell metabolism.
In a phosphoproteomic analysis of A549 cells infected with IAV, we found several viral proteins to be differentially phosphorylated during the viral life cycle. With regard to NS1 we were able to identify several phosphorylated amino acid residues, some of which were previously unknown. This prompted us to investigate the role of NS1 phosphorylation for viral replication in detail. Using reverse genetics we generated a set of influenza A/PR/8/34 (H1N1) virus mutants. These mutants encoded NS1 with phosphoacceptor sites mutated into amino acids that either mimic a constitutive phosphorylation (negatively charged residue: e.g. D or E) or that cannot be phosphorylated (e.g. A, G or L).
We analysed the mutants by investigation of viral mRNA and protein expression level as well as progeny virus titers. First data indicates that NS1 threonine (T) residue 215 (T215) is crucial for virus growth, at least in cell culture using A549 cells. Mutations resulted in attenuated virus growth. Mimicked constitutive phosphorylation (T215D mutant) strongly reduced viral titers at later time points of infection. In addition, the T215L mutation in NS1 also affected viral titers. Based on this we will further investigate the molecular mechanism leading to reduced virus replication of the NS1 T215L and T215D mutant.

Keywords: Influenza A virus, phosphoproteom, non-structural protein 1 (NS1)

P89

Comparison of the infection of porcine precision-cut lung slices by porcine influenza A virus H3N2 and porcine coronaviruses

Tanja Krimmling and Christel Schwegmann-Weßels

Institute for Virology, University of Veterinary Medicine Hannover

The porcine respiratory disease complex (PRDC) is caused by a combined infection of several viruses and/or bacteria. Porcine influenza A virus and coronaviruses can be part of these viruses infecting the porcine respiratory tract. We use the *ex vivo* system of porcine precision cut lung slices (PCLS) to analyze the infection by influenza and coronaviruses. We compared single infection by H3N2, transmissible gastroenteritis coronavirus (TGEV), and porcine respiratory coronavirus (PRCoV) – a respiratory variant of TGEV emerging in the 1980s in Belgium. PRCoV mainly causes mild disease of the respiratory tract, whereas TGEV can infect the respiratory and the gastrointestinal tract. Vital PCLS were infected 1, 2, and 3 d.p.i. and the site of infection was analyzed by confocal microscopy after staining of viral antigen. Results showed infection by TGEV and PRCoV in the PCLS, but also a different distribution of TGEV infection compared to H3N2. The influenza virus has been proven to infect ciliated and mucus-producing cells of the respiratory epithelium in the bronchioles (Punyadarsanyia et al., 2011). As TGEV has a sialic acid binding activity but no enzymatic activity like influenza virus to cleave sialic acids we will compare the effect of neuraminidase treatment on viral replication prior to infection by H3N2 and both coronaviruses. Further analysis will be done to determine the impact of an influenza and coronavirus coinfection on viral replication and distribution.

Keywords: PRDC, H3N2, coronaviruses, PCLS, neuraminidase

P90

Ribavirin prevented severe influenza caused by intra-host evolution of pandemic H1N1 influenza A virus (A(H1N1)pdm09) mpJena/5258 with hemagglutinin 222D/G (HA-222D/G) polymorphism in mice

Nora Seidel[1], Heike Braun[1], Martina Richter[1], Elisabeth Walther[1], Anja Hoffmann[1], Peter Wutzler[1], Andreas Sauerbrei[1], Michaela Schmidtke[1]

[1]Department of Virology and Antiviral Therapy, Jena University Hospital, Jena, Germany

HA-222D/G polymorphism was frequently detected in clinical A(H1N1)pdm09 isolates. Recently, we demonstrated that it facilitates viral intra-host evolution by a switch from HA-D222 to HA-G222 variants leading to severe pneumonia in mice. Here, we address the question whether this might be prevented by the neuraminidase inhibitor oseltamivir or the nucleoside analog ribavirin.
As shown before, A(H1N1)pdm09 mpJena/5258-infected, placebo-treated BALB/c mice were taken ill on day 2 p.i. Symptoms (body weight loss and clinical score) continuously worsened till day 4 p.i. After a short recovery on day 5 p.i., a second phase of symptom worsening was observed till day 8 p.i. Thereafter, surviving mice recovered.
To prevent the second, severe phase of disease, mice were treated with 100 mg/kg/d oseltamivir or ribavirin for 5 and/or 7 days (starting 4 h before infection). The effects of both drugs on survival, body weight loss, clinical score, mean time to recovery, viral titers in lung and trachea on day 4 p.i., and pneumonia were analyzed. Oseltamivir had neither an effect on viral replication in the lung on day 4 p.i. nor on parameters used to characterize the severity of influenza in mpJena/5258-infected mice. In contrast, ribavirin that significantly inhibited viral replication acted highly effective. It significantly reduced lethality, body weight loss, and clinical score.
Our results demonstrate that anti-influenza drugs inhibiting viral replication in the lung, like ribavirin, might prevent severe influenza caused by clinical A(H1N1)pdm09 isolates with HA-222D/G polymorphism because they affect viral intra-host evolution.

Keywords: antiviral mouse model, oseltamivir, ribavirin, HA polymorphism

P91

Pathogenic potential assessment of avian Influenza viruses isolated in Asian Russia

Sharshov K.A. [1,2], Sivay M.V. [1,2], Glushchenko A.V. [1], Kurskaya O.G. [1,2], Gulyaeva M.A. [2], Yurlov A.K. [3], Alekseev A.Yu. [1,2], Shestopalov A.M. [1,2], Shkurupiy V.A. [1]

[1]Research Center of Clinical and Experimental Medicine, Russian Academy of Sciences, Novosibirsk, Russia
[2]Novosibirsk State University, Novosibirsk, Russia
[3]Institute of Systematics and Ecology of Animals, Novosibirsk, Russia

Recent outbreaks of an avian-origin H5N8, H6N1, H7N9 influenza virus raise concern of the emergence of novel reassortant viruses in Eurasia and the potential threat to the human population.
Territory of Russia covering a large part of Northern Eurasia is of special interest for influenza virus ecology and evolution. The importance of this region has been confirmed by the previous outbreaks of H5N1 among wild birds since 2005. We showed circulation of different AIV among wild birds and mammals in the natural ecosystems of Northern Eurasia. Our study confirms the significant role of AIV surveillance in Russia for the Global Influenza Surveillance Network.
The report focuses on some pathogenic, epidemiological and evolutionary aspects of animal-origin influenza viruses including H5N1 isolated in Northern Eurasia in the last decade. During surveillance for AIV in Russia more than 300 AIVs were isolated and 25 different subtypes were detected. Moreover, we isolated some rare subtypes including H1N2, H8N8, H13N8, H10N7, H15N4, H16N3, LPAI H5N1. Some of them have not been detected in Russia earlier or elsewhere.
We conducted the pathogenic potential assessment for all the viruses with molecular-biological analysis and animal models. We found some viruses to be reassortant containing the segments of the different genetic lineages. We studied all basic genetic markers of biological characteristics. The report contains more detailed comparative virological, molecular, pathogenic characteristics of viruses. Some molecular-epidemiological aspects are discussed.
This study was supported by Novosibirsk Regional Government, RFBR project No. 13-04-91179-GFEN-a, USDA grant no. 58-0210-2-040F.

Keywords: influenza viruses, pathogenic potential, risk assessment, Russia

P92

Modulation of type I interferon expression in avian tracheal organ cultures after dual infection with low pathogenic avian influenza virus and *Mycoplasma gallisepticum*

Hicham Sid, Henning Petersen, Martin Ryll and Silke Rautenschlein

Clinic for Poultry, University of Veterinary Medicine, Hannover

Influenza A virus infection may modulate the susceptibility of the host to bacterial infection as demonstrated in experimental studies in mouse models. Field observations in poultry indicate that secondary infection may dominate the clinical picture of low pathogenic avian influenza virus (LPAIV) infections. In this study, we wanted to investigate the interaction and effects of dual infection with LPAIV H9N2 and *Mycoplasma gallisepticum* (MG) on avian host tissue more closely using tracheal organ cultures (TOC) of chicken and turkey. Different parameters such as ciliostasis, innate immune response, bacterial and viral counts were assessed. Compared to mono-infected groups subsequent LPAIV infection led to faster ciliostasis and increased MG replication in both turkey and chicken TOC. Surprisingly, higher viral counts were observed in dually-infected chicken but not in dually-infected turkey TOC where lower viral titers were registered. Compared to mono-infected TOC, IFN-alpha expression in dually-infected turkey and chicken TOC was significantly down-regulated at 4 and 12 hours post infection (hpi) ($p<0.05$). Similar results were observed for IFN-beta expression. Our results suggest an additive or possibly even a synergistic effect between LPAIV H9N2 and MG on the innate immune system of these avian derived tissues, which may support the observations in the field of exacerbation of clinical disease in dually infected birds.

Keywords: Dual infection, Chicken, Turkey, Type I Interferon

P93

The enzymatic head domain directs transmembrane domain changes in the influenza virus NA protein

Diogo V da Silva[1], Johan Nordholm[1], Dan Dou[1], Jeremy S. Rossman[2] and Robert Daniels[1]*

[1]Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden.
[2]School of Biosciences, University of Kent, Canterbury, CT2 7NJ, United Kingdom.
*Correspondence: robertd@dbb.su.se; +46 8 162460

Transmembrane domains (TMDs) from single-spanning membrane proteins are commonly viewed as membrane anchors for functional domains. Influenza virus neuraminidase (NA) exemplifies this concept as it retains enzymatic function upon proteolytic release from the membrane. However, we recently showed that subtype 1 NA TMDs have become increasingly polar in human strains, suggesting the TMD is under selection pressure. Here, we investigated the N1 TMD-head domain relationship by exchanging a prototypical 'old' TMD (1933) with a 'recent' (2009) more polar TMD and an engineered hydrophobic TMD. Each exchange altered the TMD association, decreased the NA folding efficiency, and significantly reduced viral replication at 37°C, but not at 33°C where NA folds more efficiently. Passaging the chimera viruses at 37°C restored viral infectivity and NA folding efficiency by selecting for NA TMD mutations that correspond with their polar or hydrophobic assembly properties. These results demonstrate that single-spanning membrane protein TMDs can influence distal domain folding and suggests the NA TMD in H1N1 viruses has become more polar to maintain compatibility with the evolving enzymatic head domain.

Keywords: transmembrane domain, neuraminidase, co-evolution, hydrophobicity, signal anchor sequence

P94

Combined Activity of Oseltamivir and Some Biological Response Modifiers Against Experimental Infection With Influenza Virus Type A/H3N2 *in Vivo*

Lora Simeonova[1], Galina Gegova[1], Elitsa Pavlova[2], Kirilka Todorova[1]

[1]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]University of Sofia "St. Kliment Ohridski", Sofia, Bulgaria

Influenza is often associated with serious complications and huge medical and economical losses to the mankind. Targeting different aspects of the pathogenesis could be a reasonable approach to combat the severity of the disease. We investigated combined activity of oseltamivir – specific anti-influenza inhibitor and some promising biological response modifiers as stimulants enhancing the host responses against the toxic effects of viral replication in the organism. Mice were inoculated i.n. with influenza A/Panama/2007/99 (H3N2) virus 4h after first oseltamivir dose and the course lasted five days.The antioxidant ellagic acid (EA) was applied orally once daily for five days starting 2 hours prior to challenge. The immune modulator isoprinosine (ISO) was administered for 10 days in two intakes the daily dosage beginning from the day of infection. Polyphenol complex (PC) from *Geranium sanguineum* was given once 3 hours pre-infection i.n. Mortality rates, protection index (PI), mean survival time (MST) and body weight changes were determined through 14 days p.i. Beneficial effects were observed of double combinations of oseltamivir of 1.25, 2.5 and 5 mg/kg with PC (2.5 nd 5 mg/kg), EA (500 mg/kg) and ISO (500 mg/kg) by reduction of mortality rates and PI up to 90% for selected combinations. MST was prolonged up to 13.7 days and body weight loss was reduced. Data suggest that oseltamivir at daily doses lower than optimal effective one *in vivo* when administered in combination with antioxidants and immune modulators demonstrated enhanced protective effect against infection with influenza virus A (H3N2) in mice.

Keywords: influenza, antivirals, biological response modifiers, combination

P95

Surveillance for Avian Influenza Viruses in the Western Siberia (Russia), 2008-2013.

Mariya V. Sivay[1,2], Kirill A. Sharshov[2], Aleksander M. Shestopalov[1,2].

[1]Novosibirsk State University, Russia, 630090, Novosibirsk, Pirogova Str.2
[2]FGBI Scientific center of experimental and clinical medicine SB RAMS, 630117, Novosibirsk, Timakova Str. 2

The study describes the surveillance for avian influenza viruses in Western Siberia, Russia. The territory of Western Siberia (Russia) is situated in the center of Eurasian continent. This area crosses by three major migration routes – Central Asia Flyway, East Africa West Asia Flyway, and Black Sea/Mediterranean Flyway, combining bird populations from Asia, Africa and Europe. The peculiar geographic location favours the virus persistent, evolution, and climate conditions promote to maintain AIVs in water and soil for longer periods of time. Moreover, the first highly pathogenic avian influenza (HPAI) H5N1 epizootics in the Russian Federation occurred at the end of July 2005 in the Western Siberia.
During surveillance for AIV in Western Siberia, 2008-2013, more than 5000 samples from wild birds were collected. The specimens were tested for AIV by standard methods. 130 AIVs were isolated and 13 different subtypes were detected. All the isolated viruses belong to low pathogenic. Moreover, we isolated some rare subtypes (H8N8, H15N4, H16N3) which have not been detected in the Western Siberia earlier or elsewhere (according to databases). Phylogenetic analysis of different genes shows closer relationship of Russian viruses with strains from different European, African, Asian countries and from Australia.
The outcome of this study has shown the importance of Western Siberia territory in distribution and evolution of AIVs due to ecological relationships of bird populations of Russia and different Asian, European, African countries and Australia.

Keywords: Avian Influenza Virus, Surveillance, Russia

P96

Cellular RNA editing enzyme ADAR1 interacts with Influenza A virus proteins during infection and enhances virus replication.

Nikki Smith[1], Artur A Arikainen[2], Helen Wise[1], Julian Hiscox[3], Paul Digard*[1]

[1]The Roslin Institute, The University of Edinburgh, Easter Bush, Edinburgh, UK
[2] University of Cambridge, Cambridge, UK
[3]Institute of Infection and Global Health, University of Liverpool, Liverpool, UK
*Corresponding author: Paul Digard

Cellular RNA editing enzymes adenosine deaminase acting on RNA 1 (ADAR1) and ADAR2 have been shown to act as pro-viral factors for several viruses, including HIV-1, measles virus and Kaposi sarcoma associated herpes virus. ADAR enzymes are RNA binding proteins which catalyse the conversion of adenosine to inosine in double stranded RNA. Evidence of non-specific editing of Influenza and measles virus RNA has been reported, although the significance of this editing is unknown. Here we show that ADAR1 interacts with components of the viral polymerase and the NS1 protein. Influenza infection causes the re-localisation of ADAR1 from the nucleoplasm to the nucleolus and we have shown that NS1 is both necessary and sufficient to induce this re-localisation. Mutant NS1 proteins which cannot bind to RNA or interact with TRIM25 are unable to induce the nucleolar re-localisation of ADAR and this correlates with the inability of these mutants to supress the activation of the type I interferon transcription factor IRF3. ADAR1 knock-down reduces Influenza A virus replication suggesting that although ADAR1 is an interferon inducible gene, it acts as a pro-viral factor during Influenza A virus infection.

Keywords: Influenza, ADAR, editing, nucleolus, interferon

P97

Influenza A/H3N2 virus isolated in Siberia (Asian part of Russia), 2008-2013.

I. Sobolev[1], O. Kurskaya[1], T. Ilyicheva[2], A. Durymanov[2], A. Shestopalov[1].

1) State Research Center of Clinical and Experimental Medicine, Novosibirsk, Russia.
2) State Research Center of Virology and Biotechnology "Vector", Koltsovo, Russia.

Influenza virus causes frequent epidemics and periodic pandemics. The main reason for this relates to the ability of the influenza virus to overcome the immunity induced by prior influenza infection or vaccination. This is due to the high antigenic variability of surface glycoproteins of influenza virus. It is obvious that the constant surveillance of influenza activity is required.
The aim of this study is to analyze the epidemiology and surface glycoproteins variability of A/H3N2 influenza virus in Siberia, 2008-2013. Siberia region occupies an area of about 13.1 million km². The population numbers 40 million that is more than 27% of Russia population. Influenza virus strains under our study were isolated in different settlements (major cities) of Siberia region separated by hundreds of kilometers from each other. Clinical samples were collected from patients with ILI-symptoms within 3 days after illness onset. Sample collection was performed since October of 2008 to June of 2013. In total 3393 samples from Asian part of Russia were analyzed.
Totally 531 influenza virus strains (15,6%) in MDCK cell culture were isolated: 262 (49%) strains belonged to influenza A/H3N2 viruses, 101 (19%) – to influenza A/H1N1 and 168 (32%) strains – to influenza B viruses. For further study 29 strains of influenza virus A/H3N2 from different localities of the Asian part of Russia were selected. For HA and NA of selected strains analysis of the nucleotide and amino-acid changes, evolutionary variability analysis and phylogenetic analysis were performed.

Keywords: Siberia, Influenza virus, A/H3N2, Genetic analysis.

P98

Phenotypic and genotypic significance of influenza viruses identified in the Republic of Moldova

C. Spinu, Veronica Eder, P. Scofertsa, R. Cojocaru, Ig. Spinu, Ala Donos

National Center for Public Health, Chisinau, Republic of Moldova

An important segment of the clinical-epidemiological and virological surveillance system of influenza (ILI – influenza like infections), acute respiratory infections (ARI) and severe acute respiratory infection (SARI) morbidity is the results of the development and study of influenza viruses' phenotypic and genotypic properties. During the 2012-2014 period in the National Influenza Center recognized by WHO, in collaboration with Influenza Center of Institute "Cantacuzino", Bucharest and the WHO CC for Reference and Research on Influenza, National Institute for Medical Research, London were isolated in MDCK cells and MDCK - SIAT1 cultures and identified by haemagglutination inhibition test (HAI) 27 strains of influenza viruses: 10 strains of influenza A(H1N1)pdm, 6 – A(H3N2), and 11 – influenza B virus from positive in rRT-PCR samples collected from patients with presumptive clinical diagnosis "ILI", "ARI" , and "Pneumonia". Dominant influenza viruses during 2012-2013 influenza season were A(H1N1)pdm (48 %), and during 2013-2014 season - influenza A(H3N2) (95 %) viruses. Strains isolated and studied by HAI test were not significantly different from antigenic variants of reference influenza viruses and could be considered similar to A/California/7/2009 H1N1pdm and A/Texas/50/2012 H3N2 influenza viruses respectively. The phylogenetic trees (Northern Hemisphere) demonstrated that influenza viruses A(H1N1)pdm fall into genetic group 6C (common substitution D97N in gene HA1), influenza viruses A(H3N2) – genetic group 3C.3 (common substitutions N145S, V223I in gene HA1, and D158N in gene HA2 – resulting in the loss of a potential glycosylation site) and influenza B viruses – genetic groups 2 (common substitutions T121S, T75N, T181A, D196N in HA gene), and 3 (common substitutions S150I, N165Y, G229D, D196N).
It is important to note that all isolates of influenza viruses in neuraminidase inhibition assay were sensitive to neuraminidase inhibitors: Oseltamivir and Zanamivir and were similar to those included in the influenza vaccine formula recommended by WHO for 2012-2013 and 2013-2014 influenza seasons respectively.
Obtained results demonstrate that the segment regarding the highlight and evaluation of the phenotypic and genotypic properties of influenza viruses as component of the unique clinical-epidemiological and virological surveillance system of ILI, ARI and SARI morbidity has an important significance for the Republic of Moldova in the context of policy on influenza vaccine use, optimization of the treatment management and prophylaxis of mentioned infections, prognosis of the epidemic process and significant reduce of the negative impact on the health system.

Keywords: influenza virus, phenotypic, genotypic

P99

Two independent evolutionary pathways of HPAIV.

Olga Stech, Jutta Veits, Sayed Abdelwhab, Ute Wessels, Thomas C. Mettenleiter, Jürgen Stech

Institute of Molecular Virology and Cellular Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 17493 Greifswald – Insel Riems, Germany

HPAIV originate from low-pathogenic precursors by acquisition of a polybasic HA cleavage site (HACS). Beside this prime virulence determinant, additional adaptive changes might accumulate already in those precursors during their circulation in gallinaceous poultry prior to the emergence of an HPAIV. Since this evolutionary process is not well understood, we aimed to unravel the genetic determinants which, beyond the polybasic HACS, are required for transformation of LPAIV into HPAIV. To select a minimal gene constellation sufficient for high virulence, we co-transfected plasmids coding for all eight genes from an H5N1 HPAIV and seven, except HA, from an H5N1 LPAIV, and used the supernatant to infect chickens. Shed reassortants carried the HPAIV PB2, NP, HA, NA, and M genes; a reconstituted virus was highly pathogenic and transmissible like the wild-type. A tailored H5N1 reassortant, carrying the LPAIV NA, exhibited 100% lethality both in inoculated and contact chickens. The same lethal phenotype was exhibited by an LPAIV reassortant carrying only the HPAIV HA and NA. However, abolishing the NA stalk deletion led to considerably reduced lethality and no transmission. Conversely, an LPAIV reassortant carrying only the HPAIV HA but the LPAIV NA with engineered stalk deletion displayed 100% lethality both after primary or contact infection. Such virulent phenotypes require a polybasic HACS and either the adapted polymerase, M and NS genes together, or an NA with stalk deletion, indicating the existence of at least two different sets of virulence determinants and, therefore, several independent evolutionary pathways for HPAIV.

P100

Alveolar type II epithelial cells rapidly react to influenza A virus infection *in vivo* and contribute to the respiratory immune response

Sabine Stegemann-Koniszewski[1], Andreas Jeron[2], Marcus Gereke[1,2], Matthias Gunzer[3], Dunja Bruder[1,2]

[1]Helmholtz Centre for Infection Research Braunschweig
[2]Otto von Guericke University Magdeburg
[3]University Duisburg-Essen

In the lower respiratory tract, the primary targets for IAV replication are type II alveolar epithelial cells (AECII). These are increasingly recognized for their immunological potential and a number of studies have shown IAV to trigger distinct AECII responses *in vitro*. The reaction of AECII to IAV-infection within the immunological network of the lung has however not been evaluated so far.
On the first three days following infection, we isolated primary AECII from the lungs of mice infected with IAV PR8/34 and performed microarray analyses. Furthermore, we analyzed transcriptional regulation, immune cell recruitment and type I/III interferon levels in whole lung tissue to evaluate the contribution of AECII to the overall respiratory response *in vivo*.
IAV-infection triggered extensive transcriptional regulation of a multitude of genes with immunological function in AECII. The kinetic and shape of the detected response indicated AECII to react to innate viral triggers as well as soluble mediators such as type I/III interferons. Functional pathway analyses revealed that AECII were active in pathogen recognition, cell recruitment and antigen-presentation. The comparison of transcriptional regulation in AECII and lung tissue pointed at a strong contribution of AECII to respiratory anti-IAV responses.
In summary, our *ex vivo* analysis of primary murine AECII isolated from IAV-infected hosts revealed a rapid and exceptionally strong response that contributed to local host defenses. Therefore AECII need to be considered as a part of the complex immunological network of the lower respiratory tract where they integrate direct pathogen recognition with signals provided by surrounding cells.

Keywords: alveolar type II epithelial cells, gene transcriptional profiling, innate viral recognition, pulmonary host defense

P101

Optimized biological assays and pharmacophore searching for development of inhibitors of the influenza virus PA endonuclease

Annelies Stevaert[†], Salvatore Nurra[‡], Nicolino Pala[‡], Mauro Carcelli[||], Dominga Rogolino[||], Caitlin Knowlton[§], Baek Kim[§], Mercedes Alfonso-Prieto[#], Salvatore A.E. Marras[%], Mario Sechi[‡], and Lieve Naesens[†]

[†]Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[‡]Department of Chemistry and Pharmacy, University of Sassari, Sassari, Italy
[||]Department of Chemistry, University of Parma, Parma, Italy
[§]Center for Drug Discovery, Department of Pediatrics, Emory Center for AIDS Research, Emory University, Atlanta, Georgia, USA
[#]Institute for Computational Molecular Science, Temple University, Philadelphia, Pennsylvania, USA
[%]Public Health Research Institute, Department of Microbiology and Molecular Genetics, New Jersey Medical School, Rutgers University, Newark, New Jersey, USA

The influenza virus PA endonuclease, which cleaves capped cellular pre-mRNAs to prime viral mRNA synthesis, is a promising target for novel anti-influenza virus therapeutics. We evaluated a series of β-diketo acid (DKA) derivatives containing different scaffolds, and determined their structure-activity relationship in an enzymatic assay with the N-terminal part of PA (PA-Nter). We propose a pharmacophore model that defines strict structural requirements along with chemical features, to serve as the basis for design of novel PA inhibitors (PAIs) with DKA-based structures or bioisosteres thereof. Besides, we developed a molecular beacon-based PA-Nter assay which is amenable to high-throughput screening. This method also enables to compare the activity of PAIs versus Mn^{2+} or Mg^{2+}, since the catalytic site structure may slightly differ for both metal ions and Mg^{2+} appears the likely cofactor under native conditions.
Several DKA derivatives caused strong inhibition of PA-Nter with IC_{50} values comparable to that of the prototype L-742,001 (i.e. below 2 μM). Three compounds (one with a pyrrole and two with an indole scaffold) also exhibited moderate antiviral activity in cell culture (EC_{99} values: 64-95 μM), and were proven to have a dual effect by acting both upon virus entry and viral RNA synthesis (i.e. 60- to 300-fold reduction in vRNA copy number).
In order to be successful, the hit-to-lead process on novel PAIs requires rapid progression of hit compounds to relevant cell culture assays, such as the vRNP reconstitution, virus yield, and time-of-addition methods that we used to identify some relevant scaffolds for further PAI design.

Keywords: PA endonuclease, antiviral, drug design, enzymatic assay, metal dependency

P102

Adaptation of a human influenza virus to two mouse strains with different genetic backgrounds

Ruth LO Stricker[1], Sarah R Leist[1], Klaus Schughart[1,2,3]

[1]Helmholtz Centre for Infection Research, Department of Infection Genetics
[2]University of Veterinary Medicine Hannover
[3]University of Tennessee Health Service Center, USA

So far, adaptation of influenza viruses from one species to another has been studied intensively. However, it is not clear, if during the adaptation process to a new species, different virus variants become selected that depend on variant host factors within this species. We aimed to investigate, if upon adaptation of a human influenza isolate to mice a) the host genetic background influences selection of certain genetic variants, and b) different virus lineages adapted within individuals of the same mouse strain are identical. We chose C57BL/6J and DBA/2J mice for this study, whereas the first ones are known to be more resistant to an influenza infection. The human isolate A/Panama/2007/99 (H3N2) was chosen for adaptation, which is not pathogenic in mice. It was adapted as separate lineages in both mouse strains by lung-to-lung passages. The difference between body weight before infection and on day three post infection increased from passage to passage in DBA/2J mice, but not in C57BL/6J. The tenth passage of each lineage sequenced by NGS. C57BL/6J and DBA/2J mice were infected with each tenth passage and body weight and survival were monitored. This showed a broad range of virulence, from very mild to severely pathogenic, whereas DBA/2J mice exhibited more severe phenotypes. In general it was observed that in the more susceptible mouse strain DBA/2J viruses adapted faster than in a more resistant mouse strain C57BL/6J. Thus, DBA/2J mice may represent a well suitable strain for influenza virus adaptation.

Keywords: host genetic, adaptation, evolution, mice

P103

Influenza A virus HA and M2 proteins are required for normal budding and genome packaging but not for normal segmented genome assembly

Naoki Takizawa[1*], Fumitaka Momose[2], Hiyori Haraguchi[2], Yuko Morikawa[2], Akio Nomoto[1]

[1]Microbial Chemistry Research Foundation, Institute of Microbial Chemistry (BIKAKEN), Tokyo Japan
[2]Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan

The influenza A virus genome is composed of eight single-stranded negative-sense RNA segments and an influenza virus particle contains one copy of each segment. To produce the infectious virion, assembly of the segmented genome RNA, packaging of genome RNA, and budding of progeny particle need to occur correctly. However, the relation among these three steps is not well known. To reveal the relation among these three steps, we generated recombinant viruses lacking the expression of hemagglutinin (HA) or matrix protein 2 (M2) and investigated budding of progeny virion, packaging of genome RNA, and assembly of the segmented genome by electron microscopy analysis, primer extension, and immunofluorescence. The amounts of viral proteins and vRNAs collected in the supernatant from cells infected with these mutant viruses were decreased. However, no differences were observed for the segmented genome assembly between in cells infected with these mutant viruses and with wild type virus, in spite of budding defects. These results suggest that HA and M2 are not involved in the segmented genome assembly but in budding of progeny virion and packaging of genome RNA. The relation among budding, genome packaging, and the segmented genome assembly will be discussed.

Keywords: budding; genome packaging; segmented genome assembly; HA; M2

P104

Serological survey for A/H5N1, A/H6N8, A/H7N1, A/H7N7 influenza antibodies in western Georgia

D. Tsereteli, I. Karseladze, A. Machablishvili, L. Urushadze, M. Chubinidze, Kh. Zakhashvili

National Center for Disease Control and Public Health, Tbilisi, Georgia

Background: Western Georgia is located on migratory route and overwintering of wild aquatic birds. In the last years different avian influenza viruses were detected in wild birds, including A/H5N1 virus in wild swans in 2006 in that area.
Objectives: To look for evidence for A/H5N1, A/H6N8, A/H7N1, A/H7N7 influenza infection in population of western Georgia.
Methods: Totally 314 adult residents (aged 17 - 86 years, mean - 49.8, males - 55.7%, females - 44.3%) of western Georgia admitted to Zugdidi hospital during 2012-2013 were enrolled in a cross-sectional, serological study. Serum samples of participants were tested against A/H5N1, A/H6N8, A/H7N1, A/H7N7 influenza viruses by Hemagglutination Inhibition (HI) assay using Horse Red Blood cells. HI antibody titers ≥1:40 were considered as positive for evidence of previous infection.
Results: None of study subjects had HI titers ≥40 against A/H6N8, A/H7N1 and A/H7N7 influenza viruses. Only one male, 55 years, had elevated HI titers ≥40 against A/H5N1 influenza virus. The elevated titer was revealed again one year later after retesting. The man did not remember any severe respiratory infection during the last 10 years; however as a young man (80's of the twentieth century) he used to hunt for the wild birds and suffered with serious respiratory infection.
Conclusion: Detection of elevated HI titers ≥40 against A/H5N1 influenza virus in resident of western Georgia may indicate possible mild influenza infection with A/H5N1 influenza virus in the last decade or infection with A/H5N1 influenza virus in his youth related to hunting on wild birds.

Keywords: avian influenza, A/H5N1, serological study.

P105

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

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

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

To determine the composition of the influenza viral quasispecies, a very accurate and sensitive sequencing technique and standardized analysis pipeline is needed. Here we assessed the suitability of two benchtop next-generation sequencers to study the influenza quasispecies: the Illumina MiSeq (2*250 bp) sequencing-by-synthesis technique and the Ion Torrent PGM (Ion 318 chip v2, 400 bp kit) semiconductor sequencing technique. We first validated the accuracy and the sensitivity of both sequencing techniques using plasmid DNA. In general, Illumina MiSeq sequencing is the most accurate, with substitutions being the most dominant error type. The majority of Ion Torrent PGM sequencing errors were insertions and deletions (indels), mostly in homopolymer regions. To evaluate their suitability to determine the influenza quasispecies composition, influenza A PR8 virus that was grown on MDCK cells was sequenced. An optimized RT-PCR led to efficient amplification and uniform coverage of all eight genomic RNA segments. In accordance with the plasmid-derived data, more sequencing errors (majority indels) were generated by the Ion Torrent PGM than with the Illumina MiSeq. Both techniques detected the most variation in the HA segment of the virus quasispecies. Surprisingly, both techniques detected also some non-synonymous variation in the conserved PB1 and NP segments.

Keywords: influenza, next-generation sequencing, quasispecies

P106

Staphylococcus aureus inhibits influenza A virus-induced apoptosis

Andre van Krüchten[1], Carolin Klemm[1], Bettina Löffler[2], Georg Peters[2], Stephan Ludwig[1], Christina Ehrhardt[1]

[1]Institute of Molecular Virology (IMV), Center for Molecular Biology of Imflammation (ZMBE), University of Münster
[2]Institute of Medical Microbiology, University Hospital of Münster

Bacterial super-infections are a major complication in influenza diseases resulting in significant morbidity and mortality. Most of the fatal cases in the course of an influenza A virus (IAV) infection are a result of secondary pneumonia caused by different bacteria, among which *Staphylococcus aureus (S. aureus)* plays an important role.
One potent, highly regulated defense mechanism in response to invading microorganisms is the programmed cell death (apoptosis) that eliminates individual cells without inducing an inflammatory response. Unsurprisingly pathogens like IAV and *S. aureus* have evolved strategies to manipulate the host cell death machinery to increase their replication and survival. While, during ongoing infection, IAV-induced apoptosis facilitates the export of ribonucleoproteins out of the nucleus enhancing the release of progeny virions, *S. aureus* inhibits apoptosis via a significant increase in the expression of anti-apoptotic genes.
Although apoptosis is very well analysed during infections by either IAV or *S. aureus* alone, until today it is poorly understood how this process is controlled in presence of both pathogens. In this study we provide novel insights into the regulation of apoptotic mechanisms during simultaneous infection with IAV and *S. aureus*. Our data indicate that *S. aureus* is able to inhibit the IAV-induced apoptotic cellular response through decreased expression of the proapoptotic factor TRAIL resulting in a reduction of PARP cleavage. Thus, we unraveled a *S. aureus*-mediated mechanism that supports intracellular survival of the bacterium and may contribute to increased pathogenicity upon IAV and *S. aureus* super-infections.

Keywords: Influenza A virus, bacterial super-infection, *Staphylococcus aureus*, apoptosis

P107

Chitosan-based adjuvants for inactivated influenza vaccines: characteristics determine immunogenicity and protective efficacy in a preclinical setting

Y.M. Vasiliev[1], O.S. Kashirina[1], M.I. Chernikova[1], L.M. Khasanova[1,2]
[1]Mechnikov Research Institute of Vaccines and Sera, [2]Centre "Bioengineering", Moscow, Russian Federation

Influenza remains a global healthcare challenge and vaccines are the cornerstone of influenza prevention and control.
Adjuvants could enhance inactivated influenza vaccines, however effective, safe and affordable adjuvants are lacking.
Chitosan-based adjuvants for influenza vaccines have been described however most if not all of the studies published to date provide limited information on the initial chitosan substance (characteristics (e.g. molecular weight, deacetylation degree), source (e.g. crab, fungi) and purity (e.g. endotoxins, proteins)), methods used to evaluate the characteristics and prepare the final formulation (anions (e.g. glutamate, hydrochloride), pH, sterilization approaches) and/or form (e.g. solution, particles). Moreover, chitosan characteristics may alter as the final formulation is prepared. Thus, evaluation of adjuvant activity for chitosan-based formulations is currently challenging if not impossible.
Panels of adjuvants based on commercially-obtained and laboratory-produced chitosan substances with varying characteristics were studied. The initial substances and final formulations were characterized; adjuvant solutions were prepared using standardized approaches and various pH and anions. Characteristics of some commercially-obtained chitosan substances differed significantly from specifications of the manufacturers.
Groups of mice were immunized intramuscularly once and twice with various adjuvanted inactivated influenza vaccines, protective efficacy and immunogenicity (against the vaccine and drift strains by HAI and MN sera antibodies) were evaluated.
Chitosan characteristics determine immunogenicity and protective efficacy of intramuscularly administered inactivated influenza vaccines. A number of characterized chitosan-based candidates performed equally or better than well-known adjuvants.
Further studies elucidating the role of chitosan characteristics in immunogenicity, protective efficacy and mechanisms of action for influenza vaccines are urgently needed.

Keywords: influenza, vaccines, adjuvants, chitosan, immunogenicity

P108

Immunogenicity graphs as a method of assessment and comparison of viruses in the contemporary influenza vaccine

E.M. Voytsehovskaya[1]*, A.A. Vasilyeva[1], E.V.Kuznetsova[1], V.S. Vakin[1], A.A.Sominina[1]

[1]Research Institute of Influenza, Saint-Petersburg, Russia
*presenting author

A method of graphical evaluation of immunogenicity of the influenza viruses in contemporary vaccine was proposed.
About 200 sera of volunteers vaccinated in 2008-2009 were examined. In addition to traditional tests of immunogenicity evaluation of influenza vaccine (GMT, percent of seroconversions) immune response of vaccinated people was assessed by graphing picture of immunogenicity. This chart reflects rise of antibody titers (RA) and medium ratio (MR) of antibodies in sera for several groups vaccinated.
Applied to the test material charting method of immunogenicity evaluation showed greater visibility, the possibility of estimating value of the immune response and its level.
Comparison of graphs MR antibodies in volunteer groups with preexisting level of influenza antibodies allowed to conclude the potential immunogenicity of the vaccine strains within different triple vaccine. Thus, influenza virus A/Brisbane/59/07 (H1N1) RA peak registered was higher (1:64-1:256) in trivalent vaccine "B" in comparison with vaccine "A".
The differences graphs of immunogenicity of vaccine viruses with the similar antigenic structure as compound of triple vaccine were shown. At that the traditional form of the immune response evaluation remained statistically invalid (P>5%) were shown.
Form of MR graphs differed significantly for influenza virus A/Uruguay/716/07 (H3N2) in vaccines "A" and "B", while both vaccines met the CPMP criteria. Unequal immunogenic activity of this strain in the trivalent vaccine "A" and "B" was confirmed by significant differences in the rate of seroconversion in vaccinated people (61 and 75%, respectively).
Graphical presentation of immunogenicity of influenza vaccine strains can be a useful addition to common methods of their evaluation.

Keywords: graphical interpretation of immunogenicity, influenza vaccine, immunity

P109

Virulence of chimeric avian influenza viruses of subtypes H4 and H8 with polybasic cleavage site

Siegfried Weber, Jutta Veits, El-Sayed M. Abdelwhab and Thomas C. Mettenleiter

Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 17493 Greifswald – Insel Riems, Germany

Avian influenza viruses (AIV) are classified according to their pathogenicity in chickens into low pathogenic (LP), exhibiting hemagglutinins (HA) of all subtypes (H1-H16), or highly pathogenic (HP), restricted to the subtypes H5 and H7. In the latter subtypes, existence of polybasic cleavage site (PCS) within the HA is mostly the main virulence determinant. To investigate if the HP phenotype is restricted to these two subtypes, we cloned the HA genes of nearly all HA subtypes, introduced an artificial PCS and generated the respective reassortants in an HPAIV H5N1 background. The generated mutants carrying the H8, H2 or H4 HA were lethal for infected chickens. Thereafter, the role of PCS, HA and/or other segments in virulence of the LPAIV H4 and H8 in chickens were studied using reassortants of H4/H5, H8/H5 and H4/H8 exhibiting PCS motifs. The introduction of a PCS into these avirulent LPAIV caused only mild disease. Within the H4 background the introduction of the HPAIV H5 HA showed the highest increase in virulence followed by the nucleoprotein (NP). In contrast, in the H8 background a recombinant virus containing the H5 NP was more virulent than that carrying the H5 HA. Interestingly, a reassortant of H8 HA with PCS in the H4 background was also capable of causing lethal disease. Thus, our results indicated the possible emergence of non-H5/H7 HPAIV and posed the question about the role of NP in contribution to virulence. Therefore, LPAIV infections in poultry should not be underestimated.

Keywords: Avian influenza, virulence, proteolytic cleavage site, reverse genetics, chickens

P110

Immunization with Neuraminidase Deficient Influenza Virus is highly immunogenic and non-pathogenic to wild type and immunocompromised mice

Barbosa, R.P.A. 2*; Salgado, A.P.C. 1*; Garcia, C.C. 3,4; Lima, F.H.; 3,4;. Lopes, G.A.O. 3,4; Rachid, M.A. 5; Peixoto, A.C. 3,4; de Oliveira, D.B. 6; Ataíde, M.C. 2; Zirke, C.A.1; Cotrim T.A.1; Costa, E.A.1; Almeida, 6 G.M.F.; Russo, R.C. 3,4**; Gazzinelli, R.T. 1,2,7** and Machado, AM.V. 1**

*the authors contributed equally to perform the experiments; **the authors contributed equally to conceive and to design the experiments

1 Laboratório de Imunopatologia, Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, Minas Gerais, Brasil; 2 Laboratório de Imunoparasitologia; 3 Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia; 4 Laboratório de Imunologia e Mecânica Pulmonar, Departamento de Fisiologia e Biofísica; 5 Departamento de Patologia Geral, 6 Laboratório de Vírus, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil; 7 Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

Recombinant influenza viruses are promising viral platforms to be used as antigen delivery vectors. To this aim, one of the most promising approaches consists to generate recombinant viruses harboring partially truncated neuraminidase (NA) segments. To date, all studies have been pointed to safety and usefulness of this viral platform. However, some aspects of the inflammatory and immune responses triggered by those recombinant viruses and their safety to immunocompromised hosts remained to be elucidated. In the present study, we generated a recombinant influenza virus harboring a truncated NA segment (NA-Δ) and evaluated the innate and inflammatory responses and the safety of this recombinant virus to wild type or knock-out (KO) mice with impaired innate (Myd88 KO) or acquired (RAG KO) immune responses. Our results showed that recombinant influenza virus harboring truncated neuraminidase segment abrogated lung and systemic inflammatory response in wild type mice and were completely harmless to KO mice. We also demonstrated that vNA-Δ infection could prevent unbalanced cytokine production that strongly contributes for lung damage in infected mice. In addition, the recombinant influenza virus was able to trigger both local and systemic virus specific humoral and T CD8+ cellular immune responses which protected immunized mice against the challenge with a lethal dose of homologous A/PR8/34 influenza virus. Taking together, our findings indicate that the neuraminidase deficient virus results in mild lung inflammation, induces a strong protective immunity against influenza challenge and are safe even to immunocompromised hosts.

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P111

Co-infection with Dengue Virus Exacerbates Influenza Replication in Cell Culture and In Ferrets

Thomas G. Voss[1], Mei-Chun Chen[1], Shih-Chao Lin[1], Gena J. Nichols[1], Somanna K. Naveen[1], Robert W. Cross[2], Bryan S. Kaplan[3]

[1] Laboratory of Virology, SRI Internioal, Harrisonburg VA USA
[2] University of Texas Medical Branch, Galveston TX USA
[3] St. Judes Children's Research Hospital, Memphis TN USA

During the 2009 influenza pandemic, Dengue/Influenza co-infection of patients with high mortality was observed in Nicauragua. Based on these findings, we sought to explore the potential interaction of these two viral agents in cell culture and in ferrets. During co-infection of A549 cells, we noted a significant increase in influenza virus loads with a concurrent decrease in dengue virus loads compared to singly infected control cultures. We show that exacerbation of influenza replication requires prior infection with dengue (24 hours before influenza challenge), and that dually infected cells are infrequent indicating an indirect effect of the co-infecting virus on replication of the other. Further studies demonstrate inhibition of influenza-mediated apoptosis in co-infected A549 cell culture suggesting a possible mechanism for enhanced influenza virus replication in the co-infected cultures. In studies in ferrets, we have similar results with influenza replication enhanced and dengue replication inhibited after co-infection. Taken together, our early results indicate a potential role for dengue infection in enhanced influenza replication and pathogenesis in vitro and in vivo. The mechanism(s) of altered replication for each pathogen during co-infection are currently being studied in our laboratory, with a focus on the potential role of Dengue NS1 and influenza NP interactions in vitro and in vivo. Taken together, these results suggest a potential role for dengue infection in enhanced influenza replication in dengue endemic areas also circulating influenza viruses.

P112

Towards a better understanding of influenza virus nucleoprotein phosphorylation and its role in virus replication

Pieter Vrijens, Els Vanstreels, Sam Noppen, Evelien Vanderlinden, Annelies Stevaert, Sandra Liekens, Dirk Daelemans, Lieve Naesens

Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

The diverse functions of the influenza virus nucleoprotein (NP) are thought to depend on its phosphorylation at specific amino acid residues. We here focused on three conserved sites (Thr3, Ser377 and Thr472) reported to be phosphorylated in the NP of influenza A/WSN/33 (Hutchinson et al., PLOS Pathogens, 2012). These three sites were mutated to evaluate the role of their phosphorylation in influenza virus replication and polymerase activity [as assessed in a viral ribonucleoprotein (vRNP) reconstitution assay]. The reverse-engineered A/WSN/33 viruses with the NP substitutions T3A, T3S, T3E, S377A, S377N, T472A or T472E displayed similar replication fitness as wild-type (WT) virus. The S377E change in NP (which introduces a fixed negative charge) was found to be non-viable, although its polymerase activity was similar to that of WT. To study NP functionality by live-cell imaging, we tried to develop the TC-FlAsH™ technique which requires introduction of a short tetracysteine tag. We first mutated the A/WSN/33 NP motif $CLPACV_{275\text{-}280}$ into the TC-FlAsH tag CCPGCC. This change was found to be non-viable in terms of virus replication, although activity in the vRNP reconstitution assay was comparable to that of WT. Confocal microscopy revealed that the $CCPGCC_{275\text{-}280}$ motif causes inhibition of NP nuclear export. Consequently, the enhanced FLNCCPGCCMEP motif was introduced at the N-terminus of NP, but again the virus proved to be non-viable. As long as the TC-FlAsH™ assay for NP remains to be established, microscopic examination of its functions (and the role of NP phosphorylation) relies on classical methods such as anti-NP staining.

Keywords: nucleoprotein; phosphorylation; polymerase activity; live-cell imaging; TC-FlAsH™ technique

P113

Genetic characterization of an adapted pandemic 2009 H1N1 influenza virus that exhibits improved replication rates in human lung epithelial cells

Xenia C. L. Wörmann[1], Markus Lesch[1], Konstantin Okonechnikov[1], Christian Sieben[2], Andreas Herrmann[2], Andreas Geißner[3], Chakkumkal Anish[3], Thomas F. Meyer[1] and Alexander Karlas[1]

[1] Department of Molecular Biology, Max Planck Institute for Infection Biology, 10117 Berlin, Germany; [2] Department of Biology, Molecular Biophysics, Humboldt University Berlin, 10115 Berlin, Germany; [3] Max Planck Institute for Colloids and Interfaces, 14424 Potsdam, Germany

The last influenza pandemic in 2009 was caused by a swine-origin H1N1 influenza virus that was able to spread rapidly around the world. Although this variant was less pathogenic than anticipated, the threat remains that it might acquire additional mutations that could make it more virulent over time. Therefore, detailed characterization of pathogenicity factors of such newly emerged viruses is important for accurate risk assessment. To estimate the potential risk of this 2009 pandemic virus, we passaged the variant A/Hamburg/04/09 sequentially in human lung epithelial cells (A549). Strikingly, after passage six we observed a 100-fold increased virus replication rate compared to the wild type. All gene segments were sequenced by high-throughput sequencing using Illumina technology, and 5 dominant mutations could be identified. Subsequently, all detected mutations were validated using a reverse genetics approach, and all possible combinations were analyzed. The increased replication rate could clearly be pinpointed to two mutations within the hemagglutinin (HA) gene segment, whereas the other mutations had no significant influence on the viral growth rate. Interestingly, one of the identified mutations is located within the receptor binding site, the other one could be found in the stem domain of HA. Currently, we are characterizing the two relevant mutations further using hemagglutinin binding and stability assays. Taken together, our data suggest that two mutations within the hemagglutinin gene segment enable the 2009 pandemic swine-origin virus to grow to significantly increased titers, and upcoming *in vivo* studies will demonstrate their impact in an animal model.

Keywords: hemagglutinin, viral growth rate, adaptation, virus cell interaction

P114

Attachment of stearate to the HEF protein of Influenza C virus contributes to viral infectivity and affects membrane fusion activity

Mingyang Wang[1], Kai Ludwig[2], Christoph Böttcher[2], Michael Veit[1]

1 Institute of Virology, Veterinary Medicine, Free University Berlin
2 Research Center of Electron Microscopy, Department of Chemistry, Free University Berlin

The only spike protein of Influenza C virus, the haemagglutinin-esterase-fusion glycoprotein HEF combines receptor binding, receptor hydrolysis and membrane fusion activities. All haemagglutinating glycoproteins of Influenza virus are S-acylated at cysteine residues, but with different fatty acids. HAs of influenza A virus contain stearate attached to a cysteine positioned at the end of the transmembrane region, whereas two cytoplasmic cysteines contain palmitate. HA of Influenza B virus possessing two cytoplasmic cysteines contains palmitate, whereas HEF of influenza C virus having only one transmembrane cysteine is stearoylated. Several studies illuminated the essential role of S-acylation of HA for replication of Influenza A and B virus by affecting budding and/or membrane fusion, but the role of acylation of HEF was not known.
Using reverse genetics we rescued a virus containing non-stearoylated HEF, which was stable during continuous passages and showed no competitive fitness defect. However, the growth kinetics of the mutant virus was reduced by one log. Acylation of HEF does neither affect the kinetics of its plasma membrane transport nor the amount of HEF incorporated into virus particles. Electron microscopy showed that the shape of viral particles and the hexagonal array of spikes typical for Influenza C virus were not influenced by this mutation indicating that virus budding was not disturbed. Interestingly, hemolysis assays revealed that the extent and kinetics of membrane fusion were reduced in mutant virus, especially at suboptimal pH values. Ongoing studies will show whether acylation of HEF affects lipid-mixing (hemifuison) or opening of a fusion pore.

Keywords: Influenza C virus, reverse genetics, HEF, acylation, membrane fusion

P115

Human-adaptive influenza virus mutation PB2 627K contributes to evasion of nucleocapsid recognition by RIG-I

Michaela Weber[1], Hanna Sediri[1], Ina Binzen[1], Sebastian Bänfer[2], Ralf Jacob[2], Linda Brunotte[3], Adolfo García-Sastre[5,6,7], Silke Stertz[4], Veit Hornung[8], Georg Kochs[3], Martin Schwemmle[3], Hans-Dieter Klenk[1], Friedemann Weber[1]

[1]Institute for Virology, Philipps-University Marburg, D-35043 Marburg, Germany, [2]Department of Cell Biology and Cell Pathology, Philipps-University Marburg, 35043 Marburg, Germany, [3]Department of Virology, University Freiburg, 79008 Freiburg, Germany; [4]Institute for medical Virology, University Zürich, 8057 Zürich, [5]Department of Microbiology, [6]Department of Medicine, Division of Infectious Diseases, and [7]Global Health and Emerging Pathogens Institute, Mount Sinai School of Medicine, New York, New York 10029, USA,[8]Institute for Clinical Chemistry and Clinical Pharmacology, University Hospital, University of Bonn, 53127 Bonn, Germany

Influenza viruses represent a significant health risk for humans. To restrict virus replication and spread within the host, a rapid antiviral response is crucial. In our project, we aimed to identify the first step of influenza virus infection inducing antiviral defense mechanisms.
The cytoplasmic DExD/H box RNA helicase RIG-I plays a key role in sensing RNA viruses for innate immunity. For the cytoplasmic Bunyaviruses, our group has recently shown that RIG-I is capable to respond to 5` triphosphorylated (5`ppp) double-stranded RNA packaged into nucleocapsids, the first viral structure to enter the host cell.
Applying a diverse array of methods led us to the conclusion that RIG-I recognizes also incoming virus nucleocapsids of influenza A virus (FLUAV) during their short cytoplasmic transit to the nucleus. RIG-I activation was mediated by 5'ppp dsRNA on nucleocapsids and modulated by an adaptive mutation of the polymerase subunit PB2. Avian FLUAV nucleocapsids possessing a mammalian-restricted PB2 polymerase subunit (627E) displayed an increased RIG-I sensitivity as compared to the mammalian-adapted PB2-627K. Both markers of PB2-627E in mammals, decreased polymerase activity and reduced nucleocapsid assembly, were substantially restored in RIG-I-defective cells.
These results identify RIG-I as a sensor of incoming FLUAV nucleocapsid and as a mammalian restriction factor for FLUAV with the avian PB2-627E. We propose that RIG-I acts independent of its IFN signaling capacity by directly promoting nucleocapsid disassembly to impair the viral life cycle immediately upon virus entry.

Keywords: RIG-I, innate immunity, influenza virus, immune evasion

P116

Depletion of NK cells lead to opposed phenotypes in mice after influenza A infection

Esther Wilk, Leonie Dengler, Klaus Schughart

Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, University of Veterinary Medicine, Hannover and University of Tennessee Health Science Center, Memphis, USA

The role of different immune cell populations in context of the flu disease is an important topic of ongoing research. Recent publications revealed different results regarding the impact of natural killer (NK) cells on the outcome of influenza A infections in murine models. To study the role of NK cells, we depleted this population by using different antibodies (α-asialo, αNK1.1 and αCD122) and used two different NKp46 (*Ncr1*) mutant mouse strains. After infection with different influenza A viruses (H1N1, H3N2, H7N7) we monitored body weight loss and survival. Most notably, we observed an ambivalent role of NK cells. *E.g.* in mice infected with the virus causing higher pathogenicity (PR8F) that usually succumb to the infection, the depletion had a positive effect with slower body weight loss and higher survival rates. In contrast, the depletion of NK cells after infection with PR8M, the variant with lower virulence and 100% survival lead to increased susceptibility and decreased survival. In conclusion, a beneficial or adverse role of NK cells in fighting influenza A infections depends on the subtype and virulence of the virus.

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Serologic screenings for avian influenza A (H7N9) among high-risk groups in the early stage of H7N9 circulation in Guangdong Province, China

Jie Wu[1], Lirong Zou[1], Lei Pei[2], Xianqiao Zeng[1,3], Hanzhong Ni[1] , Lijun Liang[1] , Haojie Zhong[1], Yinchao Song[1], Xin Zhang[1] , Jinyan Lin[1], Changwen Ke[1]

[1]Center for Disease Control and Prevention of Guangdong Province, China, 511430
[2]International Dialogues and Conflict Management, Vienna, Austria
[3]Gangdong Provincial Institute of Public Health, China

Background: To study the prevalence of the novel avian influenza A (H7N9) virus among high risk groups in the early stage of epidemics in Guangdong Province located in the southern part of China, we conducted retrospective serologic studies among samples collected from individuals who had close contacts with the first H7N9 infected patient reported in Guangdong Province, those who most likely exposed to the first H7N9 infected poultry, and those who might expose to H7N9 in the environmental setting .
Methods: Three different strains of H7N9 virus isolated from human, poultry and environment, which are commonly found in the avian influenza virus circulation, were selected as the reference strains to conduct the serological study. The H7N9 specific antibodies were screened among the blood specimens collected from the 561 individuals who had contacts with the patient, contacts with the infected poultry, or with possible environmental exposures. A modified horse red-blood-cell hemagglutinin inhibition (HAI) was employed to do the preliminary screening.
Results: The first H7N9 infected patient and contaminated environment in Guangdong Province were detected with Real-time PCR(RT-PCR) and H7N9 strains were isolated with eggs and sequenced. The alignment result indicated that the antigenic sites of the viruses from three different sources (poultry, human and environment) were highly conserved. With 3 viruses from different origins as probing antigen, all the HAI titers from the 561 samples was less than 20, and no specific antibodies against any of the three selected H7N9 strains were detected from all these 561 samples.
Conclusions: Our findings suggest that in the early circulating of H7N9 virus in Guangdong Province, the antigenic sites of HA protein of H7N9 strains from different hosts were highly conserved. The transmission capacity of cross-species of H7N9 is very limited, and the latent infection rate is very low.

Keywords: Influenza virus; H7N9; antibody; serologic screening

P118

Comparative genomic analysis of 4 novel H7N9 virus strains isolated in Guangdong province, 2013

Xian-Qiao Zeng[1, 2], Da-Wei Guan[1], Chang-Wen Ke[1], Li-Rong Zou[1], Han-Zhong Ni[1], Li-Jun Liang[1], Ling-ling Lang[2], Xin Zhang[1], Ying-Chao Song[1], Jin-Yan Lin[1], Jie Wu[1*]

[1]Institute of Microbiology, Center for Disease Control and Prevention of Guangdong Province, Guangzhou, China
[2]Guangdong Provincial Institutes of Public Health, Center for Disease Control and Prevention of Guangdong Province, Guangzhou, China
[*]Corresponding authors: Jie Wu

Background: As the biggest province in south China, Guangdong province is both one of the stop-over in Asian-Australian migratory birds' flyway and trade center with thriving poultry trading. Although in the first strike of H7N9 (29th March to 8th May) no human infected case of novel H7N9 was identified, the province is still in high risk given the short geographic distance over the epidemic region.
Methods: To understand the probability of novel H7N9 outbreak, we have sequenced the whole genomes of four novel H7N9 strains including two poultry market isolates and two human infected viruses.
Results: Phylogenetic analyses indicate that the two human infected viruses, highly similar in sequence, were likely to originate from poultry market like ancestor strains and evolve to infect human through re-assorting with local avian H9N2 virus. And Pearl River delta, mainly Shenzhen, Huizhou and Dongguan, was estimated as high risk region considering the isolated locations of the two human infected viruses. Significantly, we have identified signature amino acid PB2-627E in the second human infected virus A/Guangdong/2/2013(H7N9), which has been previously described in human infected H9N2 strains in Hong Kong. Likely, this signature amino acids is associated with mild symptom displayed by both H7N9 infected 3-years-old child in Dongguan city and H9N2 infected 5 years old child in Hong Kong.
Conclusion: In summary, comparative genomic analysis of 4 novel H7N9 virus strains in Guangdong province identified multiple segments re-assortment with local H9N2 and a signature PB2-627E associated with mild symptom in the human infected novel H7N9 viruses. Given this, strict monitoring and frequent surveillance of the novel avian-origin H7N9 virus are still urgently needed so as to prevent the potential outbreak in the province.

Keywords: genomic analysis, H7N9 virus, assortment

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Continuing reassortment leads to the genetic diversity of Influenza H7N9 in Guangdong China (submitted by Jie Wu)

Jin Lu[1,2], Xianqiao Zeng [1,2], Dawei Guan[1], Lirong Zou[1], Lina Yi[1, 2], Lijun Liang[1], Hanzhong Ni[1], Min Kang[1], Xin Zhang[1], Haojie Zhong[1], Xiang He[1, 2], Corina Monagin[3], Jinyan Lin[1] and Changwen Ke[1#]

[1]Guangdong Provincial Center for Disease Control and Prevention, No. 160, Qunxian Road, Panyu District, Guangzhou, China.
[2]Guangdong Provincial Institution of Public Health, Guangdong Provincial Center for Disease Control and Prevention, No. 160, Qunxian Road, Panyu District, Guangzhou, China
[3]Metabiota Inc., One Sutter, Suite 600, San Francisco CA 94104 USA

On March 30, 2013, a novel avian influenza A H7N9 virus causing severe human respiratory infections was identified in China. Preliminary sequence analyses have shown that the virus is a reassortant of H7, N9 and H9N2 avian influenza viruses. In this study, we conducted enhanced surveillance for H7N9 virus in Guangdong, China, from April to August 2013. We isolated two H7N9 viral strains from environmental samples associated with poultry markets and one from a clinical patient. Sequence analyses showed that the Guangdong H7N9 virus isolated from April to May shared high sequence similarity with other strains from eastern China. The A/Guangdong/1/2013(H7N9) virus isolated from the Guangdong patient on the 10th of August 2013 was divergent from other previously sequenced H7N9 viruses and more closely related to local circulating H9N2 viruses in the NS and NP genes. Phylogenetic analyses revealed that four internal genes NS, NP, PB1 and PB2 of A/Guangdong/1/2013(H7N9) were in different clusters with previously identified H7N9 viruses in other provinces of China. The discovery here implicates that continuing reassortment led the A/Guangdong/1/2013(H7N9) viral emergence as a novel H7N9 virus in Guangdong China, and that viral adaptation to avian and human hosts must be assessed.

Keywords: reassortment; influenza virus; H7N9 virus

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Streptococcus suis **affects the replication of swine influenza virus in porcine tracheal cells.**

Nai-Huei Wu[1], Fandan Meng[1], Maren Seitz[2], Peter Valentin-Weigand[2], Georg Herrler[1]

[1]Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany
[2]Institute of Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

Swine influenza viruses (SIV) are important pathogens affecting pigs of all ages. Secondary infection by *Streptococcus suis* may enhance the severity of disease in piglets infected by SIV resulting in huge economic losses. To date, the relationship between SIV and *S. suis* still remains unclear. In this study, we established an *in vitro* co-infection model based on newborn pig trachea cells (NPTr). Two SIV variants A/sw/Bad Griesbach/IDT5604/2006 H1N1 and A/sw/Herford/IDT5932/2007 H3N2 were used to compare subtype differences. Our previous studies showed that this H3N2 strain had a higher replication rate and induced a strong ciliostatic effect in pig precision-cut lung slices, while the H1N1 strain only had a mild effect on the ciliary activity. Wild type *S. suis* (WT) and an noncapsulated mutant strain (Δcps) were selected as secondary bacterial pathogens. NPTr cells were first inoculated with SIV, followed by bacterial inoculation. The course of infection was monitored by immunofluorescence microscopy and by determining the virus titers at different time points. After SIV infection, the adhesion rate of WT bacteria was enhanced. Different from Δcps, most of the WT bacteria adhered to SIV infected cells. Furthermore, the viral replication rates of both H1N1 and H3N2 SIV were reduced when cells were co-infected by *S. suis* WT strain. Interestingly, the virus titers in WT co-infected groups were ten-fold lower than in SIV mono-infection or Δcps co-infected groups at 24 h.p.i. These results indicate that *S. suis* and SIV affect each other in the infectious behavior in our NPTr cell model.

Keywords: swine influenza viruses, *Streptococcus suis*, co-infection

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The adaptation of avian influenza viruses to the respiratory epithelium of pigs

Wei Yang[1], Fandan Meng[1], Darsaniya Punyadarsaniya[2], Markus Hoffmann[1], Juergen Stech[3], Dirk Hoeper[3], Martin Beer[3], Christel Schwegmann-Wessels[1], Xiaofeng Ren[4], Georg Herrler[1]

1. Institute of Virology, University of Veterinary Medicine, Hannover, Germany
2. Institute of Virology , Mahanakorn University of Technology, Bangkok, Thailand
3. Friedrich Loeffler Institut, Bundesforschungsinstitut für Tiergesundheit, Greifswald, Germany
4. College of Veterinary Medicine, Northeast Agricultural University, Harbin, China

Pig is an important host for influenza A viruses and may play a crucial role in the interspecies transmission. To analyze the infection by influenza viruses, we have established precision-cut lung slices (PCLS) from the porcine lung as a culture system for differentiated respiratory epithelial cells. In PCLS, the differentiated epithelial cells are maintained in their original setting. As differentiated respiratory epithelial cells are the primary target cells for influenza virus infections, PCLS provide an interesting system to analyze the adaptation of avian influenza viruses to the respiratory epithelium of pigs. Avian influenza viruses of the H9N2 subtype were subjected to several passages in PCLS. Adaptation of the avian viruses to growth in porcine cells was evident in a shortening of the growth cycle. Sequence analysis revealed that few amino acid changes occurred during the different virus passages. The importance of the individual mutations is currently analyzed by generating recombinant viruses that contain the respective mutated proteins. Our study will help to understand the processes involved in the adaptation of H9N2 influenza viruses to new hosts.

Keywords: respiratory epithelium, precision-cut lung slices, avian influenza viruses, mutation

P122

Host-specific regulation of M segment expression via the non-structural (NS1) protein of influenza A virus

M Zheng, P Wang, WJ Song, XF Huang, BWY Mok, SY Lau, Siwen liu and H Chen

Department of Microbiology, Li Ka Shing Faculty of Medicine, HKU

Cross-species transmission of animal influenza A viruses to humans may lead to a pandemic. There are lots of host restriction for cross species transmission including receptor binding specificity, host innate immunity and limitation of viral replication in host cells. In the past 15 years, sporadic human infections by avian influenza A viruses have occurred. However, little is known about how viral gene expression is regulated by exploiting host pro-viral factors during evolution. There is evidence showed that the non-structural (NS1) protein of influenza A virus may regulate the splicing of M segment during virus replication. In this study, I examined the regulation of expression of M proteins (M1 and M2) by the NS1 derived from human H1N1 (WSN) and H3N2 (HK68), and two avian (CK1 and V08) viruses in different host cells. I found that after infection in mammalian cells, the M1 or/and M2 expression was attenuated in viruses containing avian origin NS1 compared to the virus containing human origin NS1. In contrast, the M1 expression is comparable among viruses regardless of the NS1 from avian or human sources. These results suggest that M segment may be regulated by the NS1 protein in a host-specific manner and may associate with host adaptation.

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New in Influenza therapy: meter dose manual inhaler containing aprotinin, a protease inhibitor

Oleg P. Zhirnov[1], Natasha O. Bokova[2], Elena I. Isaeva[1], Irina V. Vorobjeva[1], Olga A. Saphonova[2], Nikolai A. Malyshev[2]

D.I. Ivanovsky Institute of Virology, Moscow 123098[1)], Moscow Infectious Clinics N1, Moscow 119419[2)]

Aprotinin, a natural protease inhibitor from bovine lungs is widely used in heart and brain surgery as anti-fibrinolytic drug under the trade mark Trasylol™, Contrycal™, Gordox™, etc. Notably, aprotinin was found to suppress a cleavage of influenza virus HA by host respiratory proteases and reduce virus replication in respiratory epithelium (for review see Zhirnov et al., Antiviral. Res. 92(1): 27-36 2011). Therapeutic and antiviral efficacies of inhalations of aprotinin aerosol generated either by meter dose manual (MDI) inhalers were studied in influenza patients. These clinical trials were performed during winter-spring outbreaks caused with seasonal H3N2 and pandemic Influenza H1N1pdm09 viruses. In studies with propellant A134-type MDI (Aerus™ Russia), patients inhaled nasally 2 aerosol doses of aprotinin (160 Kallikrein-inhibiting Units (KIU)) each 2 hours for 5 days. In comparison group, patients were treated with tablets of Ingavirin™ (a synthetic peptidoamine with unknown antiviral target), 90 mg per day for 5 days. On day 2 after treatment virus loads in nasal-pharyngeal washes were determined by real time PCR. About 10 fold decrease of virus load in aprotinin patients was determined in comparison to Ingavirin patients. Duration of clinical symptoms, such as rhinorrhea, weakness, headache, sore throat, cough, sore thorax, fever, was 1-2 days shorter in aprotinin then in ingavirin group. Similar therapeutic efficacy was achieved when influenza patients received 10 min inhalations 3 times daily for 5 days of aerosolized aprotinin solution generated in ultrasonic or ejector stationary nebulizers. About 500 patients were observed and no side effects and nasal-pharyngeal discomfort were documented in aprotinin-treated patients. Aerosolized aprotinin is recommended as a drug of choice against Influenza caused by different viruses, including seasonal H1N1, H3N2, swine-like H1N1pdm09, and avian-like H7N9 viruses.

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Identification of determinants in type II transmembrane serine proteases which control activation of the influenza virus hemagglutinin

P. Zmora, A.-S. Moldenhauer, S. Pöhlmann

Infection Biology Unit, German Primate Center, Göttingen, Germany

The proteolytic cleavage-activation of the influenza virus hemagglutinin (HA) by host cell proteases is essential for viral infectivity and the responsible proteases are potential targets for antiviral intervention. Recently, members of the type II transmembrane serine protease (TTSP) family, including TMPRSS2 and HAT, were shown to activate influenza viruses. Why other TTSPs do not cleave and activate HA, despite robust expression and autocatalytic activation in HA expressing cells, is at present unclear. Therefore, the aim of this study was to identify molecular determinants in TTSPs, which control the ability of these enzymes to cleave and activate HA.
We have created and functionally analysed chimeras between TMPRSS2 (which cleaves HA), and TMPRSS3 (which fails to cleave HA). For this purpose, the cytoplasmic, transmembrane, stem or catalytic domains were exchanged between these proteins. We demonstrate that the exchange of the stem region abrogates HA activation by TMPRSS2 and endows TMPRSS3 with the ability to activate HA, indicating a key role of the stem in HA activation. Immunostaining and confocal microscopy revealed that the active TMPRSS3 chimera colocalized with HA while the wt protein did not. Moreover, all HA-activating TTSPs tested colocalized with HA while inactive TTSPs did not, indicating that the cellular localization of TTSPs might be a determinant of HA proteolytic activation. These results indicate that the stem region in TTSPs can determine HA activation, potentially by controlling the cellular localization of these enzymes.

Keywords: type II transmembrane serine proteases, hemagglutinin, influenza

PARTICIPANTS

A

Adamenko, Lyubov
Novosibirsk State University
Russian Federation

Aguiar Moreira, Étori
Medical Center – University of Freiburg
Institute of Virology
Germany
etoriufv@yahoo.com.br
Poster no. 1

Ainai, Akira
National Institute of Infectious Diseases
Japan
ainai@nih.go.jp
Poster no. 3

Alymova, Irina
Center for Disease Control and Prevention
USA
xeq3@cdc.gov
Poster no. 4

Anandrao Patil, Amol
University of Münster
Institute of Molecular Virology
Germany
apatil543@gmail.com

Anastasina, Maria
University of Helsinki
Finland
maria.anastasina@helsinki.fi

Andrieux, Florian
Institut Pasteur
France
florian.andrieux@pasteur.fr

Arendt, Annika
Philipps-University Marburg
Institute of Virology
Germany
arendta@staff.uni-marburg.de

Asanuma, Hideki
National Institute of Infectious Diseases
Japan
asanuma@nih.go.jp
Poster no. 5

B

Bachmannn, Mandy
Max Planck Institute for Dynamics of Complex Technical Systems
Germany
bachmann@mpi-magdeburg.mpg.de
Poster no. 7

Bäumer, Nicole
*pro**innovera*** GmbH
Germany
baeumer@proinnovera-cro.de

Bahgat, Mahmoud
TWINCORE - Centre for Clinical and Experimental Infection Research
Germany
Mahmoud-Bahgat.Riad@helmholtz-hzi.de
Poster no. 8

Barbezange, Cyrill
Institut Pasteur
France
Poster no. 9, 10

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

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

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

Benninger, Gerlinde
University Hospital Münster
Germany
benninge@ukmuenster.de

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

Bespalowa, Julia
Justus-Liebig-University Giessen
Germany
julia.bespalowa@innere.med.uni-giessen.de

Bhide, Yoshita
University Medical Center Groningen
Netherlands
y.c.bhide@umcg.nl

Börgeling, Yvonne
University of Münster
Institute of Molecular Virology
Germany
borgelin@uni-muenster.de
Poster no. 6

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

Bradley, Konrad
Imperial College London
United Kingdom
k.bradley@imperial.ac.uk

Bragstad, Karoline
Norwegian Insitut of Public Health
Norway
karb@fhi.no

Brett, Katharina
Freie Universität Berlin
Fachbereich Veterinärmedizin
Institut für Immunologie und Molekularbiologie
Germany
katharina.brett@gmail.com
Poster no. 11

Bruchhagen, Christin
University of Münster
Institute of Molecular Virology
Germany
c_bruc02@uni-muenster.de

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

Buhl, Sabine
Quagen GmbH
Germany
Sabine.Buhl@qiagen.com

Burmeister, Miriam
University of Münster
Institute of Medical Microbiology
burmeister.miriam@web.de

C

Camilloni, Barbara
University of Perugia
Italy
barbara.camilloni@unipg.it
Poster no. 12

Cho, Michael
Iowa State University
USA
mcho@iastate.edu

Christersson-Wiegers, Anmari
University of Münster
Institute of Molecular Virology
Germany
achristersson@uni-muenster.de

Cojocaru, Radu
National Center for Public Health
Moldova
rcojocaru@cnsp.md

Connaris, Helen
University of St. Andrews
United Kingdom
hc6@st-andrews.ac.uk
Poster no. 13

Crameri, Michel
Institute of Medical Virology
University of Zurich
Switzerland
crameri.michel@virology.uzh.ch
Poster no. 14

D

Dai, Meiling
Utrecht University
Faculty of Veterinary Medicine
Virology division
Netherlands
M.Dai@uu.nl

Danilenko, Daria
Research Institute of Influenza
St. Petersburg, Russia
daria.baibus@gmail.com
Poster no. 15

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

Davina Nunez, Rocio
Robert Koch-Institute
Germany
rociodavinha@outllook.com

Daxer, Marc Andre
Heinrich-Heine-Universität Düsseldorf
Germany
marc.daxer@hhu.de

de Haan, C.A.M. (Xander)
Utrecht University
Netherlands
c.a.m.dehaan@uu.nl

de Jonge, Jorgen
National Institute of Public Health and the Environment (RIVM)
Netherlands
jorgen.de.jonge@rivm.nl

De Marco, Maria Alessandra
Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA)
Italy
mariaalessandra.demarco@isprambiente.it
Poster no. 16

de Vries, Erik
Utrecht University
Netherlands
e.devries@uu.nl

Deblanc, Celine
Anses
France
celine.deblanc@anses.fr
Poster no. 17

Delagrave, Simon
Sanofi Pasteur
USA
simon.delagrave@sanofipasteur.com

Delmas, Bernard
INRA Unite VIM
France
bernard.delmas@jouy.inra.fr
Poster no. 18

Deng, Lei
Ghent University
Belgium
lei.deng@irc.ugent.be
Poster no. 19

Dewar, Rebecca
The Roslin Institute
Edinburgh University
Scotland
Rebecca.Dewar@roslin.ed.ac.uk
Poster no. 20

Dierkes, Rüdiger
Universität Münster,
Institut für Molekulare Virologie
Germany
r_dier01@uni-muenster.de

Di Pietro, Andrea
San Raffaele Scientific Institute
Italy
Poster no. 21

Dong, Wei
University of Groningen
Netherlands
d.wei@umcg.nl

Dornfeld, Dominik
University of Freiburg
Department of Virology
Germany
dominik.dornfeld@gmx.de

Dudek, Sabine Eva
University of Münster
Institut of Molecular Virology
Germany
Sabine.Dudek@uni-muenster.de
Poster no. 22

Dudek, Alexandra
University of Freiburg
Department of Virology
Germany
alexandra.dudek@uniklinik-freiburg.de

Dürrwald, Ralf
IDT Biologika GmbH
Dessau-Roßlau, Germany
ralf.duerrwald@idt-biologika.de

Dunga, Jacob
Federal Ministry of Health
ATBU Teaching Hospital Bauchi
Nigeria
jacobdunga@yahoo.com

E

Eder, Veronica
National Center for Public Health
Moldova
creatacrea@yahoo.com

Ehrhardt, Christina
University of Münster
Institute of Molecular Virology
Germany
ehrhardc@uni-muenster.de

Eiden, Sebastian
University of Freiburg
Department of Virology
Germany
basti.eiden@gmail.com

Eropkin, Mikhail
Research Institute of Influenza
Russian Federation
eropkin@influenza.spb.ru
Poster no. 23

Eropkina, Elena
Research Institute of Influenza
Russian Federation
elena.eropkina@gmail.com
Poster no. 74

Eva Wilson, Marian
Suntreso Goverment Hospital
Ghana
marianevawilson@gmail.com

Ezeh, Chukwunonso Peter Excel
Lugansk State Medical University
Ukraine
excel4reall@gmail.com

F

Faulds, Daryl
Gemmus Pharma Inc.
USA
dfaulds@gemmuspharma.com

Ferrara, Francesca
University of Kent
Viral Pseudotype Unit
United Kingdom
ff63@kent.ac.uk
Poster no. 24, 25, 26

Fesenko, Anna
L.V. Gromashevsky Institute of Epidemiology and Infectiuos Diseases NAMS of Ukraine
Ukraine
fesna2007@rambler.ru

Fonville, Judith
University of Cambridge
United Kingdom
jmf77@cam.ac.uk
Poster no. 27

Frensing, Timo
Max-Planck-Institute Magdeburg
Germany
frensing@mpi-magdeburg.mpg.de

Fründ, Christian
Cilian AG
Germany
fruend@cilian.de

G

Gack, Michaela
Harvard Medical School
USA
Michaela_Gack@hms.harvard.edu

Gallo, Lili
Max Planck Institute for Dynamics of Complex Technical Systems
Germany
gallo@mpi-magdeburg.mpg.de

Gao, George Fu
Chinese Academy of Sciences Institute of Microbiology
China
gaof@im.ac.cn

Garcia-Sastre, Adolfo
Icahn School of Medicine at Mount Sinai
USA
Adolfo.Garcia-Sastre@mssm.edu

Garcia, Cristiana
Federal University of Minas Gerais (UFMG)
Brazil
criscoutog@yahoo.com.br
Poster no. 29

Gerber, Marie
CNRS
France
m.gerber@ibmc-cnrs.unistra.fr
Poster no. 30

Gieselmann, Lutz
University of Münster
Institute of Molecular Virology
Germany
l_gies04@uni-muenster.de

Götz, Veronika
University of Freiburg
Germany
veronika.goetz@uniklinik-freiburg.de
Poster no. 28

Goncharova, Elena
Institute of Chemical Biology and
Fundamental Medicine, SB RAS
Russian Federation
goncharova-ep@rambler.ru
Poster no. 31

Gossens, Annabel
University of Münster
Institute of Medical Microbiology
a_goss02@uni-muenster.de

Graf, Laura
University Medical Centre Freiburg
Institute of Virology
laura.graf@uniklinik-freiburg.de

Gulyaeva, Marina
Novosibirsk State University
Russian Federation
mgulyaeva@gmail.com
Poster no. 32

Guo, Hongbo
Utrecht University
Faculty of Veterinary Medicine
H.Guo@uu.nl

Guriev, Vladimir
National Center for Public Health
Moldova
vguriev@cnsp.md

H

Haecker, Hans
St. Jude Children's Research Hospital
USA
hans.haecker@stjude.org

Hafezi, Wali
University of Münster
Institute of Medical Microbiology
hafezi@uni-muenster.de

Haghparast, Alireza
Ferdowsi University of Mashhad
Iran
alireza.haghparast@gmail.com

Hale, Benjamin
MRC-University of Glasgow,
Centre for Virus Research
United Kingdom
ben.hale@glasgow.ac.uk
Poster no. 33

Hall, Kevin
Goethe Universität Frankfurt am Main
Germany
Hall@em.uni-frankfurt.de

Haller, Otto
University Medical Center Freiburg
Insitute for Virology
Germany
otto.haller@uniklinik-freiburg.de

Hartmann, Marcus
Cilian AG
Germany
hartmann@cilian.de

Hashish, Emad Abdelsalam
Zagazig University
Faculty of veterinary medicine
Egypt
emadhashish@hotmail.com

Hassan, Ebrahim
Heinrich-Pette Institut für Experimentelle Virologie
Germany
ebrahim.hassan@hpi.uni-hamburg.de

Hatesuer, Bastian
Helmholtz Center for Infection Research
Germany
bastian.hatesuer@helmholtz-hzi.de
Poster no. 34

Hausmann, Nadine
University of Münster
Institute of Molecular Virology
Germany
n_haus01@uni-muenster.de

Heiner, Monika
University of Giessen Lung Center
Germany
Monika.Heiner@innere.med.uni-giessen.de

Heldt, Stefan
Max Planck Institute for Dynamics of Complex Technical Systems
Germany
heldt@mpi-magdeburg.mpg.de

Hernandez-Vargas, Esteban
Helmholtz Centre for Infection Research
Germany
Esteban.Vargas@helmholtz-hzi.de
Poster no. 35

Herrera Rodriguez, Jose
University of Groningen Medical Center
Netherlands
j.herrera.rodriguez@umcg.nl

Herrler, Georg
Stiftung Tierärztliche Hochschule Hannover
Germany
georg.herrler@tiho-hannover.de

Hesterkamp, Thomas
Deutsches Zentrum für Infektionsforschung
Germany
thomas.hesterkamp@helmholtz-hzi.de

Hippenstiel, Stefan
Charité - Universitätsmedizin Berlin
Department of Internal Medicine/Infectious Diseases and Pulmonary Medicine
Berlin
Germany
stefan.hippenstiel@charite.de

Hoang, Hang
Helmholtz Center for Infection Research
Braunschweig
Germany
hang.hoang@helmholtz-hzi.de
Poster no. 36

Hocke, Andreas
Charité Berlin
Germany
andreas.hocke@charite.de

Hoffmann, Julia
Heinrich Pette Institute
Germany
julia.hoffmann@hpi.uni-hamburg.de
Poster no. 37

Hoffmann, Anja
Jena University Hospital
Germany
Anja.Hoffmann@med.uni-jena.de
Poster no. 38

Holzberg, Magdalena
University of Münster
Institute of Molecular Virology
Germany
holzberg@uni-muenster.de

Homeier, Timo
Friedrich-Loeffler-Institut
Germany
timo.homeier@fli.bund.de
Poster no. 39

Honda, Ayae
Hosei University
Japan
Poster no. 40

Hrincius, Eike
St. Jude Children's Research Hospital
Department of Infectious Diseases
USA
Eike.Hrincius@StJude.org

Huang, Xiaofeng
University of Hong Kong
Hong Kong
stevehxf@hku.hk
Poster no. 41

Huckriede, Anke
University Medical Center Groningen
Netherlands
a.l.w.huckriede@umcg.nl
Poster no. 42

Huetter, Julia
Max Planck Institute for Colloids and Interfaces
Germany
Julia.huetter@mpikg.mpg.de
Poster no. 43

Hussain, Saira
University of Edinburgh
Roslin Institute
Scotland
saira.h0213@gmail.com

I

Iorio, Anna Maria
University of Perugia
Italy
annaiorio42@gmail.com

J

Jankauskaite, Lina
University of Giessen Lung Center
Germany
Lina.Jankauskaite@innere.med.uni-giessen.de
Poster no. 44

Jansen, Friederike
German Research Platform for Zoonoses
c/o University of Münster
Germany
friederike.jansen@ukmuenster.de

Jasim, Seema
Roslin Institute
University of Edinburgh
Scotland
seema.jasim@roslin.ed.ac.uk
Poster no. 45

K

Kallfass, Carsten
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
Germany
carsten.kallfass@bvl.bund.de

Karlas, Alexander
Max-Planck-Institute for Infection Biology
Germany
karlas@mpiib-berlin.mpg.de

Kathum, Omer
University of Münster
Institute of Molecular Virology
Germany
omerath@yahoo.com

Kellam, Paul
Wellcome Trust Sanger Institute
United Kingdom
pk5@sanger.ac.uk

Killip, Marian
University of St Andrews
United Kingdom
mjk3@st-andrews.ac.uk
Poster no. 48

Klemm, Carolin
University of Münster
Institute of Molecular Virology
Germany
klemmc@uni-muenster.de
Poster no. 49

Klingen, Thorsten
Heinrich-Heine University Düsseldorf
Germany
thorsten.klingen@uni-duesseldorf.de

Klinkhammer, Jonas
University Medical Center Freiburg
Institute for Virology
jonas.klinkhammer@uniklinik-freiburg.de
Poster no. 50

Kochs, Georg
University of Freiburg
Department of Virology
Germany
georg.kochs@uniklinik-freiburg.de

Konovalova, Nadezhda
Research Institute of Influenza
Russian Federation
konovalova_nadya@mail.ru
Poster no. 51

Krammer, Florian
Icahn School of Medicine at Mount Sinai
USA
florian.krammer@mssm.edu

Krimmling, Tanja
Tierärztliche Hochschule Hannover
Germany
Tanja.Krimmling@gmail.com

Kühn, Joachim
University of Münster
Institute of Medical Microbiology
kuehnj@uni-muenster.de

Kühn, Nora
Helmholtz Zentrum für
Infektionsforschung
Germany
nora.kuehn@helmholtz-hzi.de
Poster no. 46

Kühnl, Alexander
University of Münster
Institute of Medical Biochemistry
Germany
alexander.kuehnl@uni-muenster.de
Poster no. 47

Kumar, Prateek
University of Göttingen
Germany
singh_prats31@yahoo.co.in

Kumari, Rashmi
International Centre for Genetic
Engineering & Biotechnology,
India
rashmi.sharma1211@gmail.com
Poster no. 52

Kummer, Susann
University Hospital Heidelberg
Germany
susann.kummer@med.uni-heidelberg.de
Poster no. 53

Kupke, Sascha Young
Max Planck Institute for Dynamics of Complex Technical Systems
Germany
kupke@mpi-magdeburg.mpg.de

L

Lanz, Caroline
University of Zurich
Switzerland
lanz.caroline@virology.uzh.ch
Poster no. 54

Lapuente, Dennis
Ruhr-Universität Bochum
Germany
dennis_lapuente@web.de
Poster no. 55

Laske, Tanja
Otto-von-Guericke-Universität Magdeburg
Bioprocess Engineering Group
laske@mpi-magdeburg.mpg.de
Poster no. 56

Latvala, Sinikka
National Institute for Health and Welfare
Finland
sinikka.latvala@thl.fi
Poster no. 57

Legros, Nadine
University Hospital Münster
Institute of Hygiene
Germany
nadine.legros@ukmuenster.de
Poster no. 58

Leibenko, Liudmyla
L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases
Ukraine
Poster no. 59

Leist, Sarah R.
Helmholtz Centre for Infection Research
Germany
Sarah.Leist@helmholtz-hzi.de

Leite Dantas, Rafael
University of Münster
Institute of Molecular Virology
Germany
r_leit02@uni-muenster.de

Lesch, Markus
Steinbeis-Center for Systems Biomedicine Falkensee
Germany
lesch@mpiib-berlin.mpg.de
Poster no. 60

Liang, Yuhong
Icahn School of Medicine at Mount Sinai
USA
yuhong.liang@mssm.edu

Liedmann, Swantje
University of Münster
Institute of Molecular Virology
Germany
s.liedmann@gmx.net

Lindh, Erika
University of Helsinki
Finland
erika.lindh@helsinki.fi

Lopes, Antonio
University of Glasgow
Centre for Virus Research
Scotland
a.lopes.1@research.gla.ac.uk
Poster no. 61

Loregian, Arianna
University of Padova
Italy
arianna.loregian@unipd.it

Ludwig, Stephan
University of Münster
Institute of Molecular Virology
Germany
ludwigs@uni-muenster.de

M

M. Abd El-Whab, El-Sayed
Friedrich-Loeffler-Institut
Germany
sayed.abdel-whab@fli.bund.de
Poster no. 2

Mäkelä, Sanna
National Institute for Health and Welfare
Finland
sanna.makela@thl.fi
Poster no. 62

Magar, Linda
University of Freiburg
Institute of Virology
linda.magar@uniklinik-freiburg.de

Malyshev, Nicolay
Infections Diseases Hospital N1
Russian Federation
info@ikb1.mosgorzdrav.ru

Manchanda, Himanshu
Hans Knoell Institute
Germany
bioinfo.himanshu@gmail.com

Mantovani, Giancarlo
University of Perugia
Italy
mantovani@pg.infn.it

Marc, Daniel
Institut National de la Recherche Agronomique (INRA)
Centre de Recherches Val de Loire
France
daniel.marc@tours.inra.fr
Poster no. 64, 65

Martinpott, Florian
Medical Virology Gießen
Germany
f.martinpott@web.de

Masemann, Dörthe
University of Münster
Institute of Molecular Virology
Germany
d_mase01@uni-muenster.de
Poster no. 66

McCullers, Jonathan
The University of Tennessee Health Science Center
USA
jmccullers@uthsc.edu

Meng, Fandan
University of Veterinary Medicine Hannover
Germany
mengfandan11111@hotmail.com
Poster no. 67

Michaelis, Martin
University of Kent
United Kingdom
M.Michaelis@kent.ac.uk
Poster no. 68

Milliron, Hsiaoyun
Max Planck Institute for Dynamics of Complex Technical Systems
Germany
milliron@mpi-magdeburg.mpg.de

Mironenko, Alla
L.V.Gromashevsky Institute of Epidemiology and Infectious Diseases
Ukraine
miralla@ukr.net

Miyake, Yukari
Hosei University
Japan
yukari.miyake.2p@stu.hosei.ac.jp

Mögling, Ramona
Erasmus MC
Netherlands
r.mogling@erasmusmc.nl
Poster no. 63

Moncorge, Olivier
Imperial College London
United Kingdom
o.moncorge@imperial.ac.uk
Poster no. 69

Mooren, Kyra
Heinrich-Heine Universität Düsseldorf
Germany
kyra.mooren@uni-duesseldorf.de

Mostafa, Ahmed
Justus Liebig University Giessen
Institute of Medical Virology
Germany
ahmed.m.elsayed@bio.uni-giessen.de
Poster no. 70, 71

Mouthaan, Justin
RIVM
Netherlands
justin.mouthaan@rivm.nl

Müther, Barbara
University Hospital Freiburg
Institute of Virology
Germany
barbara.muether@uniklinik-freiburg.de

Müthing, Johannes
University of Münster
Institute for Hygiene
Germany
jm@uni-muenster.de

N

Nacken, Wolfgang
University of Münster
Germany
nacken@uni-muenster.de

Nettekoven, Matthias
F. Hoffman-La Roche AG
Switzerland
matthias.nettekoven@roche.com

Nicholls, John
Imperial College London
United Kingdom
Poster no. 72

Nigg, Patricia
University of Zurich
Switzerland
nigg.patricia@virology.uzh.ch
Poster no. 73

Nilsson, Benjamin
University of Oxford
Sir William Dunn School of Pathology
United Kingdom
benjamin.nilsson@path.ox.ac.uk

Nitzsche, Katja
University of Münster
Institute of Molecular Virology
Germany
katjanitzsche@web.de
Poster no. 75

Nordholm, Johan
Stockholm University
Department Biochemistry & Biophysics
Sweden
johann@dbb.su.se
Poster no. 76

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

Osterrieder, Klaus
Freie Universität Berlin
Institute of Virology
no34@cornell.edu

P

Patzina, Corinna
Uniklinik Freiburg
Department of Virology
Germany
corinna.patzina@uniklinik-freiburg.de
Poster no. 77

Pavel, Victoria
*pro**innovera*** GmbH
Germany
pavel@proinnovera-cro.de

Peeters, Ben
Central Veterinary Institute of Wageningen University and Research Centre
Netherlands
ben.peeters@wur.nl
Poster no. 78

Peng, Yousong
Hunan University
College of Information Science and Engineering
China
pengyousong2012@gmail.com

Perez Giron, Jose Vicente
Heinrich Pette Institut
Germany
vicente.perez@hpi.uni-hamburg.de

Pessler, Frank
TWINCORE - Centre for Clinical and Experimental Infection Research
Germany
Poster no. 79

Petersen, Henning
University of Veterinary Medicine Hannover
Clinic for Poultry
Germany
henning.petersen@tiho-hannover.de

Phiri, Bruno Stephen July
Central Veterinary Research Institute (CVRI)
Zambia
julypondayapa@yahoo.com

Planz, Oliver
University of Tübingen
Germany
oliver.planz@uni-tuebingen.de

Pleschka, Stephan
Justus-Liebig-University Giessen
Institute of Medical Virology
Germany
stephan.pleschka@mikro.bio.uni-giessen.de

Pohl, Marie
University of Zurich
Switzerland
pohl.marie-theres@virology.uzh.ch
Poster no. 80

Preuße, Matthias
Helmholtz-Zentrum für Infektionsforschung GmbH
Germany
Matthias.Preusse@helmholtz-hzi.de

Prokopeva, Elena
Novosibirsk State University
Russian Federation
ellap@bk.ru
Poster no. 81

Q

Quang, Trong-Hung
University Hospital Münster
Germany
tronghungquang@yahoo.de

Quantius, Jennifer
Justus-Liebig-Universität Giessen
Germany
Jennifer.Quantius@innere.med.uni-giessen.de

R

Radchenko, Larysa
L.V. Gromashevsky Institute of Epidemiology and Infectious Disesases
Ukraine
larysa_rad@ukr.net

Rashid, Muhammad
Universität Münster
Institut für Molekulare Virologie
Germany
m_rash01@uni-muenster.de

Rautenschlein, Silke
University of Veterinary Medicine Hannover
Clinic for Poultry
Germany
Silke.Rautenschlein@tiho-hannover.de

Resa-Infante, Patricia
Heinrich-Pette-Institut
Leibniz Institute for Experimental Virology
Germany
patriciaresainfante@gmail.com
Poster no. 82

Rescher, Ursula
University of Münster
Institute of Medical Biochemistry
Ursula.Rescher@ukmuenster.de

Reuther, Peter
University of Freiburg
Department of Virology
Germany
peter.reuther@uniklinik-freiburg.de
Poster no. 83

Rheinemann, Lara
University of Freiburg
Institute for Virology
Germany
lara.rheinemann@uniklinik-freiburg.de
Poster no. 84

Riegger, David
University Medical Center Freiburg
Institute of Virology
Germany
David.Riegger@uniklinik-freiburg.de

Robb, Nicole
University of Oxford
United Kingdom
n.robb1@physics.ox.ac.uk

S

Sachse, Christine
Cilian AG
Germany
sachse@cilian.de

Sänger, Sandra
Robert Koch-Institute
Germany
saengers@rki.de
Poster no. 85

Sandrin, Virginie
Roche Pharmaceutical Research and Early Development Discovery & Clinical Virology Infectious Diseases DTA
Switzerland
virginie.sandrin@roche.com

Samir Ahmed, Mohamed
TWINCORE, Center for Experimental and Clinical Infection Research
Germany
mohsamir1984@yahoo.com
Poster no. 86

Saxena, Latika
University of Delhi
India
bhardwaj.latika@gmail.com
Poster no. 87

Scheiner, Christian
Cilian AG
Germany
scheiner@cilian.de

Scheller, Nicoletta
Institute for lung research
Germany
nicoletta.scheller@uni-marburg.de

Schilling, Mirjam
University of Freiburg
Department of Virology
Germany
mirjam.schilling@uniklinik-freiburg.de

Schloer, Sebastian Maximilian
University of Münster
Institute of Medical Biochemistry
SebastianMaximilian.Schloer@ukmuenster.de

Schmid, Isabell
German Research Platform for Zoonoses
c/o University of 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

Schneberger, Sebastian
Cilian AG
Germany
sebastian@schneberger.com

Schotsaert, Michael
Icahn School of Medicine at Mt Sinai
USA
michael.schotsaert@mssm.edu

Schräder, Tobias
University of Münster
Institute of Molecular Virology
Germany
tobias.schraeder@uni-muenster.de
Poster no. 88

Schreiber, Andre
University of Münster
Institute of Molecular Virology
Germany
andre.schreiber@uni-muenster.de

Schughart, Klaus
Helmholtz-Zentrum für Infektionsforschung
Germany
Klaus.Schughart@helmholtz-hzi.de

Schuler-Lüttmann, Susanne
University of Münster
Institute of Medical Microbiology
susanne.schuler-luettmann@ukmuenster.de

Schwegmann-Weßels, Christel
University of Veterinary Medicine Hannover
Institute for Virology
Germany
christel.schwegmann@tiho-hannover.de
Poster no. 89

Schwemmle, Martin
University Freiburg
Department of Virology
Germany
martin.schwemmle@uniklinik-freiburg.de

Seidel, Nora
Jena University Hospital
Department of Virology and Antiviral Therapy
Germany
nora.seidel@med.uni-jena.de
Poster no. 90

Selter, Helga
Regierungspräsidium Tübingen
Germany
helga.selter@rpt.bwl.de

Selvakumar, Balachandar
Justus Liebig University Giessen
Germany
balachandar.selvakumar@innere.med.uni-giessen.com

Sharshov, Kirill
Russian Academy of Sciences Research Center of Clinical and Experimental Medicine
Russian Federation
sharshov@yandex.ru
Poster no. 91

Shestopalov, Alexander
Novosibirsk State University
Russian Federation
shestopalov2@ngs.ru

Shi, Tianlai
F. Hoffman-La Roche AG
Switzerland
tianlai.shi@roche.com

Shin, Dai-Lun
Helmholtz Centre for Infection Research
Germany
dls12@helmholtz-hzi.de

Sid, Hicham
University of Veterinary Medicine Hannover
Clinic for Poultry
Germany
Hicham.Sid@tiho-hannover.de
Poster no. 92

Silva, Diogo
Stockholm University
Sweden
diogo@dbb.su.se
Poster no. 93

Simeonova, Lora
Bulgarian Academy of Sciences
Stephan Angeloff Institute of Microbiology
Bulgaria
losimeonova@gmail.com
Poster no. 94

Sinhadri, Balaji
Freie University Berlin
Germany
balaji23@zedat.fu-berlin.de

Sitnik, Siarhei
University of Münster
Institute of Molecular Virology
Germany
siarhei.sitnik@uni-muenster.de

Sivay Mariya
Novosibirsk State University
Russian Federation
sivaym@hotmail.com
Poster no. 95

Smith, Nikki
University of Edinburgh
Roslin Institute
Scotland
nikki.smith@roslin.ed.ac.uk
Poster no. 96

Sobolev, Ivan
State Research Center of Clinical and Experimental Medicine
Russian Federation
sobolev_i@hotmail.com
Poster no. 97

Spijkers, Sanne
National Institute for Public Health and the Environment
Netherlands
sanne.spijkers@rivm.nl

Spinu, Constantin
National Center for Public Health
Moldova
cspinu@cnsp.md
Poster no. 98

Spinu, Igor
National Center for Public Health
Moldova
ispinu@cnsp.md

Spitaels, Jan
Inflammation Research Center
VIB – UGent
Belgium
jan.spitaels@irc.vib-ugent.be

Stäheli, Peter
University Medical Center Freiburg
Germany
peter.staeheli@uniklinik-freiburg.de

Stech, Jürgen
Friedrich-Loeffler-Institut
Germany
juergen.stech@fli.bund.de
Poster no. 99

Stegemann-Koniszewski, Sabine
Helmholtz Centre for Infection Research
Germany
sabine.stegemann-koniszewski@helmholtz-hzi.de
Poster no. 100

Stertz, Silke
University of Zurich
Switzerland
stertz.silke@virology.uzh.ch

Stevaert, Annelies
Rega Institute for Medical Research
Belgium
annelies.stevaert@rega.kuleuven.be
Poster no. 101

Stricker, Ruth
Helmholtz Centre for Infection Research
Germany
ruth.stricker@helmholtz-hzi.de
Poster no. 102

T

Takizawa, Naoki
Microbial Chemistry Research Foundation
Institute of Microbial Chemistry
Japan
takizawan@bikaken.or.jp
Poster no. 103

Teixeira, Mauro M.
Universidade Federal de Minas Gerais
Brazil
mmtex.ufmg@gmail.com

Tenbusch, Matthias
Ruhr-University Bochum
Germany
matthias.tenbusch@rub.de

Tomar, Jasmine
University of Groningen
Netherlands
j.tomar@rug.nl

Touoyem, Valery Justin
Lugansk Regional Children's Hospital
Ukraine
touoyemv@yahoo.fr

Tsereteli, David
National Center for Disease Control and Public Health
Georgia
Dr.Tsereteli@gmail.com
Poster no. 104

Turkington, Hannah
MRC-University of Glasgow
Centre for Virus Research
Scotland
h.turkington.1@research.gla.ac.uk

V

Vallbracht, Melina
University of Münster
Institute of Medical Microbiology
m_vall04@uni-muenster.de

van den Hoecke, Silvie
VIB – UGent
Belgium
silvie.vandenhoecke@irc.vib-ugent.be
Poster no. 105

van Dijken, Harry
National Institute for Public Health and the Environment
Netherlands
harry.van.dijken@rivm.nl

van Krüchten, Andre
University of Münster
Institute of Molecular Virology
Germany
andre.van.kruechten@uni-muenster.de
Poster no. 106

Vasiliev, Yuri
Mechnikov Research Institute of Vaccines and Sera
Russian Federation
yuri.vasiliev@hotmail.com
Poster no. 107

Vasilyeva, Anastasiya
Research Institute of Influenza
Russia
nastukas@mail.ru
Poster no. 108

Vazquez – Armendariz, Ana Ivonne
University of Giessen Lung Center
Germany
Ana.I.Vazquez-Armendariz@innere.med.uni-giessen.de

Veits, Jutta
Friedrich-Loeffler-Institut
Germany
Poster no. 109

Verhelst, Judith
VIB - UGent
Belgium
judith.verhelst@dmbr.ugent.be

Vicenzi, Elisa
Ospedale San Raffaele
Italy
vicenzi.elisa@hsr.it

Vollmeister, Evelyn
Philipps University Marburg
Germany
evelyn.vollmeister@uni-marburg.de

Voss, Thomas G.
SRI International
Center for Immunology and Infectious Diseases
United States
thomas.voss@sri.com
Poster no. 111

Voytsehovskaya, Elena
Influenza Research Institute
Russian Federation
elenavoicex@gmail.com

Vrijens, Pieter
REGA Institute
Belgium
Pieter.vrijens@rega.kuleuven.be
Poster no. 112

W

Wang, Mingyang
Freie Universität Berlin
Institute of Virology
Germany
wangmingzang@zedat.fu-berlin.de
Poster no. 114

Warnking, Kathrin
University of Münster
Institute of Molecular Virology
Germany
kathrin.warnking@uni-muenster.de

Weber, Michaela
Philipps-University Marburg
Institute of Virology
webermi@students.uni-marburg.de
Poster no. 115

Wilk, Esther
Helmholtz Centre for Infection Research
Germany
esther.wilk@helmholtz-hzi.de
Poster no. 116

Wirth, Dagmar
Helmholtz Centre for Infection Research
Germany
dagmar.wirth@helmholtz-hzi.de

Wörmann, Xenia C. L.
Max Planck Institute for Infection Biology
Germany
woermann@mpiib-berlin.mpg.de
Poster no. 113

Wu, Jie
Guangdong Provinical Centre for Disease Control and Prevention
China
wuxiaorong@yahoo.com
Poster no. 117, 118, 119

Wu, Nai-Huei
University of Veterinary Medicine
Hannover
Institute for Virology
Germany
Nai-Huei.Wu@tiho-hannover.de
Poster no. 120

Y

Yamauchi, Yohei
ETH Zurich
Switzerland
yohei.yamauchi@bc.biol.ethz.ch

Yang, Wei
University of Veterinary Medicine
Hannover
Germany
yangwei0727@hotmail.com
Poster no. 121

Z

Zell, Roland
Universitätsklinikum Jena,
Institut für Virologie und Antivirale
Therapie
Germany
Roland.Zell@med.uni-jena.de

Zheng, Min
University of Hong Kong
Hing Kong
minzheng2010@gmail.com
Poster no. 122

Zhirnov, Oleg
The D.I.Ivanovsky Institute of Virology
Russia
zhirnov@inbox.ru
Poster no. 123

Zmora, Pawel
German Primate Center
Germany
pzmora@dpz.eu
Poster no. 124

SPONSORS

Thank you for financial support and sponsoring