Nationale
Forschungsplattform
für Zoonosen

International

# Workshop

## Zoonotic Poxviruses – An Emerging Threat?

May 13, 2011 – Berlin

Conference volume

Funded by
Federal Ministry
of Education
and Research

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# WELCOME NOTES

## Welcome

The scientific committee and the National Platform for Zoonoses invite you to attend the international workshop **Zoonotic Poxviruses – An Emerging Threat?** on May 13, 2011 in Berlin, Germany. Despite intensified research, little is known about poxvirus diversity. Still unknown poxviruses as well as unstudied animal reservoirs are discussed as important disease threats. To intensify our research efforts on zoonotic poxviruses we need interdisciplinary collaborations of virologists, immunologists, and ecologists.

This international workshop aims to combine and exchange knowledge of different research areas concerning zoonotic poxviruses. The meeting provides an unique opportunity for young poxvirus researchers to present their data to a knowledgeable audience, discuss zoonotic aspects of poxviruses, meet colleagues, and initiate collaborations. Graduate students and postdocs will present their work as short oral presentations, which were selected from submitted abstracts.

# GENERAL INFORMATION

**Scientific committee and organization**

Martin Beer | Greifswald – Insel Riems

Ingo Drexler | München

Andreas Nitsche | Berlin

Gerd Sutter | München

B. Karsten Tischer | Berlin

**Venue**

Robert Koch Institute

Nordufer 20

13353 Berlin (Wedding)

**Offical language**

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

**Meals**

Lunch will be provided at the venue as indicated in the programme.

**Contact**

National Research Platform for Zoonoses

c/o Institute of Molecular Virology (IMV)

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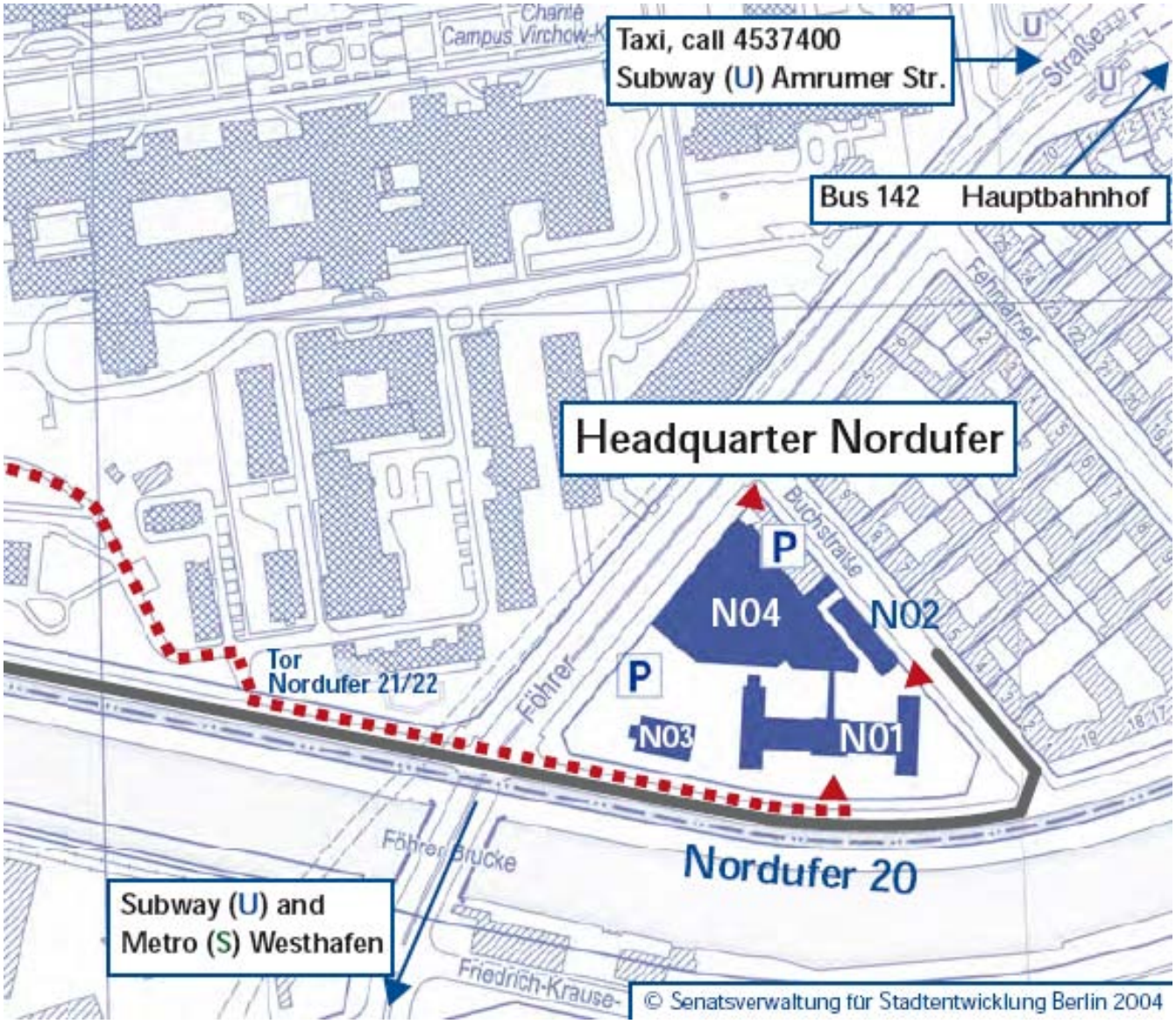
# VENUE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# PROGRAMME

# Final programme
April 29, 2011

# Zoonotic Poxviruses – An Emerging Threat?

Nationale Forschungsplattform für Zoonosen

## Friday, May 13, 2011

9.00 **Arrival / Reception**

10.00 **Welcome**
Andreas Nitsche, Berlin

10.15 **Session I – Molecular Virology**
Chair: B. Karsten Tischer, Berlin

Cell biology of vaccinia virus genome uncoating and replication | Jason Mercer, Zurich

Going BAC to characterize the impact of cowpox virus ankyrin repeat proteins on the inhibition of NF-κB | B. Karsten Tischer, Berlin

Generation of a cowpox virus deletion mutant for determination of host-range factor CP77 function | Livia Schuenadel, Berlin

Comparative analysis of the cellular gene expression profile of vaccinia virus and cowpox virus infected cells | Daniel Bourquain, Berlin

Proteome analysis of vaccinia virus IHD-W infected HEK 293 cells with 2-dimensional gel electrophoresis and MALDI-PSD-TOF MS of on solid phase support N-terminally sulfonated peptides | Jörg Doellinger, Berlin

Genomewide analysis of epidemic cowpox virus infections in Germany | Aleksandar Radonic, Berlin

12.00 – 13.00 Lunch Break

13.00 **Session II – Immunology**
Chair: Ingo Drexler, Munich

Type I interferons in poxvirus infection | Zoe Waibler, Langen

Modulation of the interferon response by avian poxviruses | Michael A. Skinner, London

Tattoo immunization with non-replicating viral vector MVA induces long-term protective immunity as well as skin-resident CD8+ T-cell memory | Andreas Muschaweckh, Munich

Spatiotemporal pattern of antigen processing during Modified Vaccinia Virus Ankara infection | Yi Zhang, Munich

Critical role of T cell immunity for rapid protective vaccination in murine model of human smallpox | Melanie Kremer, Munich

Respiratory Ectromelia virus infection of C57BL/6 mice allows detailed analysis of orthopoxvirus pathogenesis | Asisa Volz, Munich

14.45 – 15.15 Coffee Break

15.15 **Session III – Epidemiology, Clinical Virology**
Chair: Andreas Nitsche, Berlin

Monkeypox virus outbreak in the United States | Victoria Olson, Atlanta

The emergence of vaccinia virus in Brazil | Giliane de Souza Trindade, Belo Horizonte

Experiences with the laboratory investigations of clinical specimens obtained from outbreaks of suspected human monkeypox in the Democratic Republic of the Congo (DRC) | Hermann Meyer, Munich

Rats as potential reservoir for cowpox virus infections | Astrid Puppe, Berlin

Cowpox virus: characterization of an old but emerging zoonosis | Bernd Hoffmann, Greifswald-Insel Riems

Immunoglobulin libraries for the selection of recombinant antibodies against orthopox viruses | Ulrike S. Diesterbeck, Göttingen

Establishment and validation of a competitive ELISA for the retrospective diagnosis of orthopoxvirus infections in exotic zoo animals | Daniel Stern, Berlin

17.30 **Informal Discussion / Press Workshop**
Chair: Martin Beer, Greifswald-Insel Riems

Andreas Nitsche | Berlin
Victoria Olson | Atlanta
Giliane de Souza Trindade | Belo Horizonte
Gerd Sutter | Munich

18.00 **Closing Remarks**
B. Karsten Tischer, Berlin

# ORAL PRESENTATIONS

# Molecular Virology

## Friday, May 13, 2011

*Chair: B. Karsten Tischer, Berlin, Germany*

**Lecture: Cell biology of vaccinia virus genome uncoating and replication**
*Jason Mercer, Zurich, Switzerland*

**Going BAC to characterize the impact of cowpox virus ankyrin repeat proteins on the inhibition of NF-κB**
*B. Karsten Tischer, Berlin, Germany*

**Generation of a *cowpox virus* deletion mutant for determination of host-range factor CP77 function**
*Livia Schuenadel, Berlin, Germany*

**Comparative analysis of the cellular gene expression profile of vaccinia virus and cowpox virus infected cells**
*Daniel Bourquain, Berlin, Germany*

**Proteome analysis of vaccinia virus IHD-W infected HEK 293 cells with 2-dimensional gel electrophoresis and MALDI-PSD-TOF MS of on solid phase support N-terminally sulfonated peptides**
*Jörg Doellinger, Berlin, Germany*

**Genomewide analysis of epidemic cowpox virus infections in Germany**
*Aleksandar Radonić, Berlin, Germany*

**Cell biology of vaccinia virus genome uncoating and replication**

Jason Mercer
ETH Zurich, Institute of Biochemistry, Schafmattstr. 18, 8093 Zürich, Switzerland

Viruses as inert obligatory intracellular parasites rely on cellular machinery for all aspects of their lifecycle, from entry to egress. In turn, viruses have emerged as valuable tools for studying cellular processes such as endocytosis, intracellular trafficking and sorting, signaling and cytoskeletal rearrangement. We have used vaccinia, a prototypic poxvirus and model system for smallpox, to investigate the complex interactions between viruses and their host cells. Using high-throughput siRNA screening technology we assessed the role of 7,000 host cell factors in viral infection. Over 200 cellular factors required for successful vaccinia virus infection were identified. Using a variety of cell biological, virological, and microscopy techniques we further characterized one subset of these factors in regards to the viral lifecycle. We found that the ubiquitin-proteasome, which serves to degrade old and unwanted proteins, is required for productive viral infection. We further demonstrate that the proteasome is needed for productive DNA replication, late gene expression, and viral production. Release of the genome from the viral core is a pre-requisite of viral DNA replication and late gene expression. As such, our results suggest that the cellular ubiquitin-proteasome machinery is required for productive uncoating of the viral DNA. These studies provide further insight into the various cellular factors required for productive poxvirus infection. We hope to one day utilize this information for the development of cell-based anti-viral agents.

**Going BAC to characterize the impact of cowpox virus ankyrin repeat proteins on the inhibition of NF-κB**

Swaantje J. Roth, Imme Sakwa, Aiste Tamosiunaite, Zhiyoung Xu, B. Karsten Tischer
Institute of Virology, Freie Universität Berlin, Philippstr. 13, 10115 Berlin, Germany

Bacterial artificial chromosomes are versatile tools to modify large virus genomes in *Escherichia coli*. We generated a full length clone of the cowpox virus (CPXV) strain Brighton Red (BR) termed pBRF. From BAC DNA we could reconstitute virus (vBRF) in chicken embryo cells using Rabbit fibroma virus as helper virus. Thus we have a tool at hand which allows all kinds of markerless, targeted, or random sequence modification without any selection for viral function.

Our work has focused on a clade of akyrin repeat proteins (ARPs) in CPXV, proteins encoded by BR006/225, BR211 and BR220 and clustering in phylogenetic analysis. The gene product of BR006 was shown to interfere with NF-κB activation in infected cells. Also, the homologue of BR211 in Modified Vaccinia Virus Ankara (MVA) is the only functional ARP left in this attenuated virus variant after serial virus passages in chicken embryo cells. Based on pBRF we deleted the BR006/225, BR211 and BR220 either singly or in combination using markerless mutagenesis. Integrity and stability of CPXV mutants was confirmed by PCR and sequencing. Neither the single nor the triple CPXV mutant showed a significant growth difference compared to parental virus. None of the deletion mutants showed a different NF-κB/p65 localization upon induction of the pathway with TNFα. Also with NF-κB reporter cell lines we see a clear inhibition of the activation with TNFα in the parental virus as well as in all mutant CPXV clones. Our work indicates that deletion of BR006-like proteins is not sufficient to restore the NF-κB classical pathway in CPXV infected cells.

## Generation of a *cowpox virus* deletion mutant for determination of host-range factor CP77 function

L. Schuenadel[1], S. J. Roth[2], B. K. Tischer[2], A. Nitsche[1]

[1]Centre for Biological Security 1 & Consultant Laboratory for Poxviruses, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany; [2]Institute of Virology, Freie Universität Berlin, Philippstr. 13, 10115 Berlin, Germany

The host-range of *Orthopoxviruses* can either be extremely narrow, as known for *Variola virus*, or very broad, as observed for *Vaccinia virus* and *cowpox virus* (CPXV). The tropism of a poxvirus species is highly dependent on its unique repertoire of expressed host-range genes which interact with the infected cell. CPXV host-range factor CP77 was identified to be required for replication in Chinese hamster ovary (CHO) cells, but the molecular mechanism by which CP77 modulates host-range remains poorly understood.

For the elucidation of molecular events that decide whether virus replication in CHO cells is abortive or permissive, a CPXVΔCP77 deletion mutant was constructed. Therefore, a bacterial artificial chromosome (BAC) consisting of the CPXV Brighton Red genome with integrated mini-F vector sequences and the green fluorescence protein gene was used. A point mutation was introduced into the start codon of the CP77 gene by a two-step red-mediated recombination procedure followed by reconstitution of infectious virus.

Integrity of viral DNA was confirmed by whole genome sequencing. Replication kinetics of CPXV wildtype and virus expressing or lacking the CP77 protein revealed comparable *in vitro* growth properties in HEK293T cells. Infection of CHO cells with CPXVΔCP77 was abortive while CHO cells were permissive for infection with CPXV WT.

Further comparative analysis of CPXV expressing or lacking CP77 will be used for elucidation of molecular mechanisms of CP77 host-range function.

**Comparative analysis of the cellular gene expression profile of vaccinia virus and cowpox virus infected cells**

D. Bourquain, J. Hinzmann, J-W. Sim-Brandenburg, A. Nitsche
Centre for Biological Security 1, Robert Koch Institute, Berlin, Germany

Orthopoxviruses can cause disease in numerous host species including humans. Some orthopoxviruses also have the ability to infect non-natural host species and to cause zoonoses. For instance, cowpox virus and closely related vaccinia virus are both capable of establishing infection in various mammals, including humans. However, infection with vaccinia virus causes a milder disease with a reduced inflammatory response. These differences may be explained by the unique repertoire of host cell modulating factors encoded by vaccinia virus and cowpox virus, allowing productive replication in specific cells and tissues and influencing general pathogenesis.

We aimed at characterizing the specific modulation of the host cells gene expression profile by orthopoxvirus infection. In our study we analyzed changes in host cell gene expression of HeLa cells after infection with cowpox virus or vaccinia virus and compared these to each other and to the gene expression profile of non-infected cells using Agilent Whole Genome Microarray technology. Changes in the expression of selected genes were afterwards verified by TaqMan quantitative real time PCR.

We could identify major differences in chemokine gene expression in cowpox virus and vaccinia virus infected HeLa cells. Furthermore, strong induction of IL-6, IL-8 and CXCL1 secretion was identified in the cell culture supernatant following infection with cowpox virus but not after infection with vaccinia virus.

The observed differences may contribute to the greater inflammatory response towards cowpox virus infection compared to vaccinia virus infection in certain host species.

## Proteome analysis of vaccinia virus IHD-W infected HEK 293 cells with 2-dimensional gel electrophoresis and MALDI-PSD-TOF MS of on solid phase support N-terminally sulfonated peptides

J. Doellinger[1,2], S. Bartel[2], K. Darsow[2], D. Bourquain[1], R. Buchholz[2], H. A. Lange[2] and A. Nitsche[1]

[1]Robert Koch Institute, Center for Biological Security 1, Nordufer 20, 13353 Berlin, Germany; [2]Friedrich-Alexander University Erlangen-Nuremberg, Institute of Bioprocess Engineering, Henkestraße 91, 91052 Erlangen, Germany

Despite the successful eradication of smallpox by the WHO-led vaccination program, poxvirus infections continue to present a considerable health threat. The possible use of smallpox as a bioterrorist threat as well as the continuous occurrence of zoonotic poxvirus infections document the relevance to deepen the understanding of virus–host interactions. Since the permissiveness of cells for poxvirus infections is independent of cell surface receptors, but correlates with the ability of the virus to infiltrate the antiviral host response, successful infection directly depends on the host's proteome set. In this study the proteome of HEK293 cells infected with Vaccinia Virus IHD-W was analysed by 2-dimensional gel electrophoresis and MALDI-PSD-TOF MS in a bottom-up approach. The modulated expression of 24 human proteins by the infection was identified from post source decay spectra of N-terminally sulfonated peptides. The proteome analysis of infected cells provides insights into apoptosis modulation, regulation of cellular gene expression and the regulation of energy metabolism. Several of the regulated human proteins have not yet been described in correlation with poxvirus infections.

**Genomewide analysis of epidemic cowpox virus infections in Germany**

Aleksandar Radonić, Piotr Wojtek Dabrowski, Julia Tesch, Julia Wiethaus, Jung-Won Sim-Brandenburg, Andreas Kurth and Andreas Nitsche
Centre for Biological Safety 1, German Consultant Laboratory for Poxviruses, Robert Koch Institute, Berlin, Germany

Several zoonotic infections by orthopoxviruses are representing a threat to humans today. Cowpox virus (CPXV) has been enzootic in cattle in Europe; however, no such infections were diagnosed over the last decades. Instead, individual cases of CPXV infections are increasingly found in animals and humans having contact to infected animals. Both animals and humans reveal local exanthema on arms and legs or in the face. Although it is generally regarded as a self-limiting disease, immunosuppressed patients can develop lethal systemic disease that resembles a variola virus infection.
The genomes from CPXV-isolates from five humans, three rats, one cat, one beaver, one elephant, one mara, two jaguarundis and one mongoose were sequenced using massive parallel pyrosequencing. Comparing studies between the obtained genomic sequences were performed to determine the impact of changes in host range genes between the different isolates and phylogenitc relationship between the isolates.

# Immunology

## Friday, May 13, 2011

*Chair: Ingo Drexler, Munich, Germany*

**Lecture: Type I interferons in poxvirus infection**
*Zoe Waibler, Langen, Germany*

**Modulation of the interferon response by avian poxviruses**
*Michael A. Skinner, London, UK*

**Tattoo immunization with non-replicating viral vector MVA induces long-term protective immunity as well as skin-resident CD8+ T-cell memory**
*Andreas Muschaweckh, Munich, Germany*

**Spatiotemporal pattern of antigen processing during Modified Vaccinia Virus Ankara infection**
*Yi Zhang, Munich, Germany*

**Critical role of T cell immunity for rapid protective vaccination in murine model of human smallpox**
*Melanie Kremer, Munich, Germany*

**Respiratory Ectromelia virus infection of C57BL/6 mice allows detailed analysis of orthopoxvirus pathogenesis**
*Asisa Volz, Munich, Germany*

## Type I interferons in poxvirus infection

Zoe Waibler
Junior Research Group “Novel vaccination strategies and early immune responses”
Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, 63225 Langen, Germany

Type I interferons (IFNs) are pro-inflammatory cytokines, initially defined by their ability to confer resistance to viral infections. They constitute a first line of defense against many pathogenic infections and critically contribute to the initial survival of the host until the onset of adaptive immunity. Mice deficient for a functional type I IFN system are highly susceptible to infections with a broad range of different viruses including poxviruses and finally succumb to the infection. We found that upon infection of mice or cells with highly attenuated modified vaccinia virus Ankara (MVA), type I IFN responses are induced (primarily triggered by non-Toll like receptor molecules, independently of virus propagation, critically involving IFN-feedback via the IFN-receptor (IFNAR)). In sharp contrast, upon vaccinia virus (VACV) infection, type I IFN responses are inhibited. VACV-mediated IFN inhibition is a multi-step process involving secreted factors such as virus-encoded B18 protein plus intracellular components that cooperate to efficiently shut-off IFN responses. Analyzing adoptive immune responses upon poxvirus infection we showed that IFN-inducing MVA confers virus-specific $CD8^+$ T cell expansion by triggering the IFNAR on both dendritic cells and T cells. In contrast, VACV-induced T cell responses were less dependent on triggering the IFNAR. Most probably, VACV infection induces interleukin-12 that may compensate for leaking IFN and thus promotes IFNAR-independent T cell expansion. Knowing how poxviruses induce innate immune responses, how they evade from innate immune detection, and studying requirements for initiating anti-poxvirus adaptive immune responses are of great importance with respect to zoonotic infections, usage of attenuated poxviruses as vectors, and the generation of novel effective vaccines.

**Modulation of the interferon response by avian poxviruses**

Michael A. Skinner, Rebecca Robey, Stephen M. Laidlaw, Marc Davies, Io Hong Cheong
Section of Virology, Imperial College London Faculty of Medicine, St Mary's Campus, Norfolk Place, London W2 1PG, UK

Avian poxviruses represent a large and diverse group of poxviruses infecting only avian species. The diversity between the 3 major clades, representing: (i) fowlpox virus-like members, (ii) canarypox virus-like members and (iii) psittacine poxviruses, is equivalent to that seen between different genera of mammalian poxviruses. It is unlikely that avian poxviruses pose any direct threat as zoonotic agents, though they do pose a threat as emergent infections of farmed, pet, protected and reintroduced avian species. Little is known however about their host interactions in permissive species, let alone any restrictions to replication in non-permissive species. For instance, there is no indication of how fowlpox virus (FWPV) is able to resist chicken type I interferon (IFN), even though we have shown it to be highly resistant. It has none of the well known interferon modulators observed in vaccinia virus (VACV) and other mammalian poxviruses, nor does it encode proteins whose sequence suggests they might play such a role. We therefore employed genetic screens to identify FWPV-encoded modulators of chicken IFN. We identified two members of an extensive avipoxvirus gene family that target different stages of the IFN response. We are currently characterising these modulators, their mode of action and hopefully their targets.

## Tattoo immunization with non-replicating viral vector MVA induces long-term protective immunity as well as skin-resident CD8+ T-cell memory

G. Gasteiger[1,2,3], A. Muschaweckh[1], W. Kastenmuller[1,2,4], P.-A. König[1], S. Kisling[1], R. Baier[1], D. H. Busch[5], I. Drexler[1,2]

[1]Institute of Virology, Helmholtz Zentrum München; [2]Clinical Cooperation Group „Antigen-specific immunotherapy", TU München; [3]Current address: Memorial Sloan-Kettering Cancer Center, New York; [4]Current address: Lymphocyte Biology Section, Laboratory of Immunology, NIAID, NIH; [5]Institute of Medical Microbiology, Immunology and Hygiene, TU München

The skin not only provides a physical barrier to prevent invasion of pathogenic organisms but also possesses a network of specialized innate and adaptive immune cells to combat infection once this barrier has been compromised. Thus the skin represents a promising tissue for vaccine administration and the successful eradication of smallpox by inoculating vaccinia virus into the skin further underlines its potential as a vaccination target. We have developed a skin delivery method for the non-replicating viral vector modified vaccinia virus Ankara (MVA) using a tattoo device. This method allows for an effective dispersion of virus in the skin (to compensate for the lack of viral replication) and might potentially further provide an adjuvant effect by inflicting a mild skin injury. We found that despite a relatively low antigen expression at the site of infection, an MVA tattoo elicited a strong systemic virus-specific CD8+ T-cell response. MVA-tattooing also protected mice against a lethal respiratory challenge with replication-competent vaccinia virus (VACV) to a similar degree than the conventional intramuscular infection route. Moreover, delivery of MVA via tattoo efficiently recruited CD8+ T-cells to the skin, which persisted locally for at least 150 day post infection. Interestingly, given the non-replicative nature of MVA, these memory CD8+ T cells exhibit an activated phenotype that strongly resembles that of skin-resident memory T cells, which have been previously described in a latent HSV skin infection. Our data suggest that viral tattooing might be a promising vaccination strategy to induce both systemic and skin-resident immunity.

## Spatiotemporal pattern of antigen processing during Modified Vaccinia Virus Ankara Infection

Yi Zhang[1], Frank Thiele[1, 2], Ingo Drexler[1, 2]

[1]Institute of Virology, Helmholtz Zentrum München & Technische Universität München, [2]Clinical Cooperation Group „Antigen-specific immunotherapy“, Technische Universität München

Vaccinia virus (VACV), a member of the poxvirus family, is a prototype pathogen for an acute viral infection. Using the attenuated VACV-strain Modified Vaccinia Virus Ankara (MVA), we have demonstrated that primary CD8+ T cell (CTL) responses against VACV-produced antigens were dominated by cross-priming in vivo and the timing of VACV gene expression after infection has an intense impact on viral T cell epitope presentation and processing. Since knowledge about poxviral activation of CD4+ T cells is sparse, our aim was to investigate the cellular and molecular mechanisms that occur in MVA infected Bone marrow derived dendritic cells (BMDCs), that induce and shape the quality and quantity of the CD4+ T cell response.

We used recombinant MVA constructs expressing EGFP at early or late time of infection in BMDC or Hela cells as a control. We found that viral late gene product H3, involved in the virion membrane formation, was initially produced around the virus factory, and then moved to the cell membrane in the later timing of infection. Co-localization of virus factories with Golgi was seen from 5h p.i, EGFP co-localized with lysosomal structures when the protein was produced under late viral promoter control. Interestingly, infected BMDC barely phagocytosed the apoptotic debris of neighbouring infected BMDC, but activated strong CD4+ cell responses indicating direct interaction between CTL and infected professional antigen-presenting cells. These primary results encourage further research of how BMDC can process and present endogenous viral antigen on MHC II molecules and activate CD4+T cell responses.

## Critical role of T cell immunity for rapid protective vaccination in murine model of human smallpox

M. Kremer[1], Y. Suezer[2], A. Volz[1], K. Hanschmann[2], U. Kalinke[3], G. Sutter[1]
[1]Institut für Infektionsmedizin und Zoonosen, LMU Munich, Germany; [2]Paul-Ehrlich-Insitut, Langen, Germany; [3]TWINCORE, Hannover, Germany.

Vaccinations are highly effective at preventing infectious diseases but the constant threat of emerging pathogens necessitates the development of new, highly efficient vaccines that might also rapidly protect in a case of emergency. Although increasing evidence points to T cell immunity playing an important role in successful vaccination against some viral diseases, vaccine efficacy is mostly correlated to the induction of antibody responses. Here we analyze the immunological mechanism(s) of rapidly protective immunization with vaccinia virus using mousepox as an excellent surrogate model for human smallpox. Surprisingly, we found that fast protection against lethal systemic poxvirus disease solely depended on CD4 and CD8 T cell responses induced by vaccination with highly attenuated modified vaccinia virus Ankara (MVA) or conventional vaccinia virus. In contrast, selected components of the innate immune system and B cell-mediated responses were fully dispensable in preventing fatal disease through immunizations given two days before challenge. Our data clearly demonstrate that T cell immunity plays a key role in the protective efficacy of vaccination with a gold standard live viral vaccine. Moreover, rapid induction of pathogen-specific T cell responses might be critical for vaccines that need to fit a scenario of protective emergency vaccination.

**Respiratory Ectromelia virus infection of C57BL/6 mice allows detailed analysis of orthopoxvirus pathogenesis**

A. Volz[1], M. Gratz[2], Y. Suezer[2], M. Majzoub[3], K. M. Hanschmann[2], U. Kalinke[4], A. Schwantes[2], G. Sutter[1], M. Kremer[1]

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The emergence of zoonotic orthopoxvirus infections and the threat of possible intentional release of pathogenic orthopoxviruses have stimulated renewed interest in understanding orthopoxvirus infections and the resulting diseases. Ectromelia virus (ECTV), the causative agent of mousepox (MP), offers an excellent model system to study an orthopoxvirus infection in its natural host. Upon natural infection, ECTV is thought to enter the body via skin lesions. Footpad inoculation of many mouse strains allows ECTV to rapidly spread resulting in a fatal systemic disease. Interestingly, C57BL/6 mice are resistant to MP following footpad infection.

However recent studies have shown that C57BL/6 mice are susceptible for MP after oronasal uptake of ECTV. Respiratory infection of C57BL/6 mice results in a lethal systemic disease comparable to that seen after footpad inoculation of highly susceptible mouse strains. In this context, we investigated the role of the vaccinia virus ortholog N1L in ECTV infection. Respiratory infection of mice with an N1L deletion mutant virus (ECTVΔN1L) demonstrated profound attenuation of the mutant virus, confirming N1 as an orthopoxvirus virulence factor. Upon analysis of virus dissemination in vivo, we observed a striking deficiency of ECTVΔN1L spreading from the lungs to livers or spleens of infected mice. Investigating the mechanism controlling ECTVΔN1L infection, we found loss of the attenuated phenotype in mice lacking CD8+ and CD4 + cells.

Our data suggest that N1L may early interfere with innate immune responses that control systemic spread of the virus likely by influencing the adaptive cellular immune responses.

# Epidemiology, Clinical Virology

## Friday, May 13, 2011

*Chair: Andreas Nitsche, Berlin, Germany*

**Lecture: Monkeypox virus outbreak in the United States**
*Victoria Olson, Atlanta, USA*

**Lecture: The emergence of vaccinia virus in Brazil**
*Giliane de Souza Trindade, Belo Horizonte, Brazil*

**Experiences with the laboratory investigations of clinical specimens obtained from outbreaks of suspected human monkeypox in the Democratic Republic of the Congo (DRC)**
*Hermann Meyer, Munich, Germany*

**Rats as potential reservoir for cowpox virus infections**
*Astrid Puppe, Berlin, Germany*

**Cowpox virus: characterization of an old but emerging zoonosis**
*Bernd Hoffmann, Greifswald-Insel Riems, Germany*

**Immunoglobulin libraries for the selection of recombinant antibodies against orthopox viruses**
*Ulrike S. Diesterbeck, Göttingen, Germany*

**Establishment and validation of a competitive ELISA for the retrospective diagnosis of orthopoxvirus infections in exotic zoo animals**
*Daniel Stern, Berlin, Germany*

## *Monkeypox virus* outbreak in the United States

Victoria Olson
Centers for Disease Control and Prevention, 1600 Clifton Rd. Atlanta, GA 30333, USA

*Monkeypox* virus, a member of the *Orthopoxvirus* genus, causes a severe systemic infection within humans. Monkeypox was first identified in captive monkeys in 1958, and later as a human disease during the Intensified Smallpox Eradication Program led by the World Health Organization. Unlike smallpox, which is strictly a human pathogen caused by *Orthopoxvirus variola* infection, monkeypox is a zoonotic agent. Human monkeypox had only been seen within endemic regions of Africa, such as Democratic Republic of Congo, prior to 2003. In late May 2003, a child in Wisconsin was bitten by her pet prairie dog and presented with signs of human monkeypox. Subsequent epidemiologic and laboratory studies confirmed the existence of 37 cases of human monkeypox within the Midwestern United States, all who had direct contact with ill prairie dogs. Trace back studies and genetic analysis of human and animal isolates identified the source of *Monkeypox virus* as African rodents imported from Ghana, West Africa. Although the natural reservoir of *Monkeypox virus* is unknown, African rodents are the most likely candidate. The rodents were housed in proximity to captive prairie dogs, both which were subsequently sold within the exotic pet trade. The potential for establishment of *Monkeypox virus* in the United States existed, however surveillance studies have not detected the presence of virus within the wild rodent population. From our experience with the United States monkeypox outbreak and experimental studies within our laboratory, we have ascertained that the prairie dog is highly susceptible to *Monkeypox virus* and serves as an excellent small animal model for systemic *Orthopoxvirus* disease, which can also be used in evaluating potential anti-virals and vaccines.

**The emergence of vaccinia virus in Brazil**

Giliane de Souza Trindade
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Naturally occurring infections with *Orthopoxvirus* have been recognized in Brazil during the past 12 years. Infections typically occur as a zoonosis transferred from affected dairy cows to their handlers. Outbreaks have caused notable economic losses to the rural community in the vulnerable areas. Often, the entire herd, all dairy workers, and the owners from a single farm have been infected. *Vaccinia virus* (VACV) has been consistently isolated in association with the outbreaks which have had substantial impact on local economies and public health. Retrospective epidemiological data revealed that 100% of the patients develop classical poxvirus infection symptoms after direct contact with symptomatic cows. Diagnosis and reporting of human infections is often delayed or non-existent and both human and animal infections typically remain untreated. The origins of Brazilian vaccinia viruses (BVV) are unclear but previous analyses have shown that at least two distinct clades of BVV exist. The natural host of VACV remains unknown but the virus clearly persists today in Brazil. Serological analysis of wild mammals captured in wildlife preservation areas with little human presence indicated a high prevalence of animals with anti-orthopoxvirus antibodies. Although the World Health Organization (WHO) declared global smallpox eradicated in 1980, concerns over emergent poxvirus infections have increased. Considering that poxviruses affecting humans are largely zoonotic, the emergence of VACV in Brazil represents a very important model to study the epidemiology, pathogenesis, and molecular characteristics of these viruses. This understanding could also provide insights to establish ways to prevent and control poxvirus infections.

**Experiences with the laboratory investigations of clinical specimens obtained from outbreaks of suspected human monkeypox in the Democratic Republic of the Congo (DRC)**

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After the eradication of smallpox the related monkeypox virus (MPXV) has emerged as the most significant human pathogenic orthopoxvirus. The name “monkeypox” can be considered a misnomer, because the virus is maintained in African rodent reservoirs, including squirrels. Human disease clinically resembles smallpox, however person-to-person transmission is low and therefore MPXV cannot maintain itself in the human population. Studies on the burden of human monkeypox in the Democratic Republic of the Congo (DRC) were conducted by WHO from 1981 to 1986. Since then, the immunologically naïve population has increased significantly. To assess the current risk of infection, surveillance was conducted in collaboration with the NIH. Between November 2005 and November 2007, 760 laboratory-confirmed human monkeypox cases were identified. Comparison of data in the same health zone from the 1980s (0.72 per 10,000) and 2005–07 (14.42 per 10,000) suggests a 20-fold increase in human monkeypox incidence in the intervening 30 years. Nine out of ten cases occurred in individuals born after 1980, when mass smallpox vaccinations were terminated in DRC (Rimoin et al., 2010; doi: 10.1073/pnas.1005769107). Here we report the analysis of clinical specimens using a diagnostic algorithm relying on real time PCR and virus isolation. Sequencing of four genes demonstrated a striking similarity of 40 MPXV isolates analysed, however, differences in the number of repeats were noted in a genomic region located at the left terminus. The most relevant differential diagnosis was chickenpox caused by Varicella Zoster virus, accounting for almost one third of suspected cases.

**Rats as potential reservoir for cowpox virus infections**

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In the last three years the number of cowpox virus (CPXV) infections in Germany has highly increased.
CPXV is rodent borne with a broad host range. It contains the largest and most complete genome off all poxviruses, including parts with a high homology to variola virus (smallpox).
Due to several CPXV outbreaks among zoo animals and humans after contact to infected rats between 2008 and 2009, the attention regarding the transmission of CPXV through rats has thoroughly increased. Whether wild and domestic rats are a primary reservoir or just amplifying hosts for CPXV has not yet been revealed.
In the outbreaks mentioned above, domestic rats - bred on different farms throughout Europe as food for carnivores - have been identified as the source of CPXV infections.
Rats of different farms and of four different age categories (baby, small, adult, breeder) were analyzed for CPXV. Blood was tested for antibodies against CPXV by IFAT; lung and liver where tested by real-time PCR for existing CPXV-DNA.
In one farm, all animals tested showed antibodies specific against CPXV, whereas virus DNA was found in adult and breeder rats only with a prevalence of 55% and 72.8%, respectively.
Since none of the examined animals showed any pathological signs of disease, a possible asymptomatic course of a CPXV infection can be suggested. How the virus found its way into the domesticated rat population is still unknown. Nevertheless, these results indicate that rats certainly play an important role in transmitting CPXV.

**Cowpox virus: characterization of an old but emerging zoonosis**

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Cowpox virus (CPXV) infection is a zoonosis in Europe, which mainly affects pet owners or veterinarians after contact to diseased pet or zoo animals. The risk of human infection is increasing as a consequence of the decreasing immunity against orthopoxviruses in the human population since smallpox vaccination has been terminated more than 30 years ago. Most human cases of CPXV infections have been associated with infected cats, however, pet rats have become the second most common transmission host to humans in recent years. Interestingly, the young pet rats were severely ill and often died albeit wild rodent species are regarded to be the classical reservoir hosts of CPXV.

Here we report about experimental infection studies of pet rats and outbred wistar rats using three different cowpox viruses isolated 2009 and 2010 as well as the reference strain "Brighton". Wistar rats and pet rats (one isolate) were infected with $10^4$ or $10^6$ $TCID_{50}$, respectively. Viral excretion was continuously recorded by sampling oropharyngeal swabs. Additionally, the genome load of a broad set of tissues was determined. Interestingly, only a cowpox virus isolated from a diseased pet rat induced severe clinical signs and lethal infection (high dose group) both in the wistar and the pet rats, whereas the reference strain Brighton, and isolates from a cat and an alpaca caused clinical signs only in a small proportion of the inoculated animals. Furthermore, we infected cows with the "Brighton" strain or with the pet rat derived isolate. Intravenous injection, oronasal application, and scarification of the teats were the inoculation routes used, but typical cowpox lesions like blisters and crusts could be detected only in 2 out of 4 cows after intravenous inoculation.

In order to link the differences in pathogenesis to potential virulence markers we also developed a protocol for full-length viral genome sequencing. Together with the establishment of a standardised animal model and the possibility to prospectively use immunological tools, a more detailed characterization of different CPXV isolates is now possible. Identification of virulence markers by additional sequence comparison studies might be also beneficial for other zoonotic orthopoxvirus infections like monkeypox.

## Immunoglobulin libraries for the selection of recombinant antibodies against orthopox viruses

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The establishment of immunoglobulin libraries is a new approach to engineer target optimized recombinant antibodies for prophylactic, therapeutic, and diagnostic reasons. To generate recombinant human single chain (scFvs) antibodies against orthopox viruses (OPXV), we constructed an IgM-based naive library from peripheral blood lymphocytes of non-immunized blood-donors and an immunized IgG-based library from four donors vaccinated with "Dryvax®" (Wyeth Laboratories, USA). A murine anti-VACV A27L-library was prepared for diagnostic reasons. Those immunoglobulin libraries enable the selection of antibody fragments binding to any desired OPXV antigen. ScFvs are also able to influence virus replication on a subcellular level.

Immunoscreening of the human antibody libraries against VACV Elstree revealed numerous scFvs. Binding sites of several scFvs were mapped on the D8 (32 kDa) and A27 proteins (14 kDa). Differences of the kinetics to several OPXV strains resulted from amino acid residue substitutions within the target epitopes or were related to conformational changes. The binding site of an *in vitro* neutralizing scFv derived from the naive human library could not yet be identified. This scFv, however, showed 67% neutralization of a lethal dose of MPXV MSF-6 ($10^6$ pfu) applied to *Macaca mulatta* in a challenge model. Other human and murine scFvs neutralized 100 pfu of VACV Munich 1 *in vitro*. A27 and D8 specific scFvs were able to protect also *in vivo*. 100-67% of NMRI mice passively immunized intra-peritoneally with 100 µg scFv survived until day 21 p. inf. All scFvs were evaluated to work in modern diagnostic systems.

## Establishment and validation of a competitive ELISA for the retrospective diagnosis of orthopoxvirus infections in exotic zoo animals

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Various members of the genus *Orthopoxviruses* (OPV) are able to infect a broad range of different species and can cause zoonotic infections in humans. Especially cowpox virus (CPXV) has a broad host range and repeatedly caused outbreaks in zoos which led to the loss of valuable exotic animals. During a recent outbreak of CPXV at the zoo Krefeld some animals, although asymptomatic, were found to have detectable levels of antibodies against OPV.

For further studies on the seroprevalence against OPV in different zoo animals we developed a species independent competitive ELISA (cELISA) based on a biotinylated polyclonal rabbit anti vaccinia antibody.

For the validation we used a well characterized panel of 122 sera from 25 different species with known OPV sero- and infection status. The mean intra- and interassay variance of the cELISA was below 10% (9.2% intraassay-, 7.4% interassay coefficient of variation). Compared to an indirect immuonfluorescence assay (IFA) the cELISA showed a specifity of 99.99% and a sensitivity of 99.97% for unambiguous positive sera (IFA titre > 1:1280).

For exotic animals, where antibody detection by IFA was done with protein A or G, the cELISA gave more reliable results indicating that affinities of protein A or G towards immunoglobulins of certain species might hamper diagnostics. Overall, the developed cELISA as a rapid screening method of large panels is a valuable tool for species-independent retrospective seroprevalence studies of all OPV.

# POSTER PRESENTATIONS

# P1

**An siRNA-based system for the investigation of poxviral host range effects**

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*Orthopoxviruses* (OPV) are able to invade various cell types, however there is no specific cellular receptor known for OPV entry. Instead, subsequent intracellular events and the ability of OPV to modulate the host's antiviral response determine whether an infection is permissive or abortive. The OPV host range gene K1L is known to play a pivotal role in mediating *Vaccinia virus* (VACV) host tropism. K1L is essential for productive replication of VACV in rabbit kidney cells (RK13). The molecular mechanisms of K1L are poorly understood. One of its known functions is the modulation of the host's antiviral response by inhibiting NF-κB and the PKR-eIF2α-pathway. In this project, RK13 cells were transfected with plasmids expressing shRNAs that were processed intracellularly to active siRNAs against K1L. A plasmid encoded green fluorescent protein allowed an enrichment of transfected RK13 cells by fluorescence-activated cell sorting. Cell populations displayed 94 % of transfected RK13 cells. On RNA level, K1L gene expression was reduced to 20 % relative to the expression in cells transfected with a plasmid carrying a non-sense shRNA. siRNA-mediated inhibition of the replication of VACV Western Reserve was shown via indirect immunofluorescence staining, plaque assay and a novel method of impedance measuring. The results demonstrate that siRNA-based systems are an efficient alternative to the generation of deletion mutants and represent a useful tool for the investigation of poxvirus host range effects.

# P2

## Genomic expression libraries for the identification of cross-reactive Orthopoxvirus antigens

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Members of the *Orthopoxvirus* (OPV) genus are large double-stranded DNA viruses. Beside variola virus, the causative agent of smallpox, they include zoonotic pathogens like monkeypox and cowpox viruses as well as vaccinia virus, the vaccine strain used to eradicate smallpox. Since the cessation of smallpox vaccination, the occurrence of cowpox virus infections particularly affecting young non-vaccinated persons is increasing. Conventional smallpox vaccines are causing severe adverse reactions especially in individuals with impaired immunity. Therefore, new safer OPV vaccines need to be developed. Recombinant subunit vaccines are considered to be a safer alternative to conventional smallpox vaccines. For the design of effective subunit vaccines conferring protection against multiple OPV species knowledge about cross-reactive antigens is required.

In order to identify cross-reactive antigens of vaccinia and cowpox virus genomic orthopoxvirus expression libraries were constructed, validated and screened with anti-cowpox and anti-vaccinia virus sera. Through the serological screenings 21 immunogenic orthopoxvirus proteins could be identified. Among those, 16 were found to be cross-reactive between cowpox and vaccinia virus.

Serological screenings of genomic expression libraries are a powerful tool for the identification of antigenic cross-reactive proteins. The described method can also be used to find cross-reactive antigens of other OPVs or other pathogenic viruses. This knowledge could contribute to the development of safer subunit vaccines with a wider range of protection.

# P3

## Rapid and sensitive detection of Orthopoxviruses by an Abicap-immunofiltration column

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Besides the raised concern about a bioterrorist attack with smallpox virus, other members of the genus *Orthopoxvirus* (OPV) cause zoonotic infections in humans. As routine virus diagnostics depends on either expensive equipment or specially equipped laboratories with trained/skilled staff, rapid point of care diagnostics is not yet possible. To fill this gap we developed a simple, rapid and highly sensitive detection method for OPV, based on the Abicap immunofiltration method.

For that we tested polyclonal antibodies against several OPV surface proteins (A27, L1, F9, D8, H3, A33, B5) for their ability to detect viral particles. Monoclonal antibodies (mAbs) against A27 were generated and screened for sandwich ELISA compatibility. For further characterisation, affinities of the best A27 mAbs were determined by Biacore measurements. Finally, the sensitivity and cross-reactivity against different OPV strains has been evaluated on the Abicap columns.

We found that A27 was the best target for the generation of detection antibodies. The affinities of the A27 mAbs ranged between 1 and 3 nM. Rapid detection with Abicap columns was possible for all tested OPV. The limit of detection ranged from 5e3 to 1e6 PFU/ml, recombinant A27 was detectable down to 16 pg/ml.

The Abicap detection system is well suited as a first test for suspected bioterrorist samples. As other OPV strains are the cause of zoonotic infections in countries where extensive equipped laboratories are often lacking, the developed Abicap system could also be used for point of care diagnostics in these areas.

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