ICRP

1st INTERNATIONAL CONFERENCE ON RESPIRATORY PATHOGENS

GRK 1870
Bacterial Respiratory Infections

transregio 34

FRIEDRICH-LOEFFLER-INSTITUT
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

KoInfekt

Welcome note

Dear colleagues,

It is a great pleasure to welcome you to the "1st International Conference on Respiratory Pathogens (ICoRP)". This event is a joint effort of the DFG Research Training Group 1870 "Bacterial Respiratory Infection - Common and Specific Mechanisms of Pathogen Adaptation and Immune Defense", the DFG Collaborative Research Center Transregio 34 "Pathophysiology of Staphylococci in the Post-Genome-Era" and the Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health.
The "1st International Conference on Respiratory Pathogens" focusses on current research efforts in understanding co-infections of the respiratory tract caused by viruses, in particular influenza A and RSV, and bacteria, i.e. Staphylococcus aureus, Streptococcus pneumoniae and group A streptococci. Therefore, renowned speakers give a broad, up-to-date overview of this rapidly evolving and multi-disciplinary field. This event brings together an outstanding and diverse group of experts from basic and applied science from across the globe to foster the scientific exchange and to establish new contacts or collaborations.
We hope that this truly interdisciplinary event in the historic environment of Rostock, a former member of the Hanseatic League, combined with the modern aspects of a university town at the Baltic Sea is the perfect place to create a stimulating atmosphere of exciting talks, inspiring discussions, and scientific curiosity.
We are pleased to welcome you in Rostock and wish you a successful and stimulating conference.

Yours sincerely,

Prof. Dr. rer. nat.
Sven Hammerschmidt

University of Greifswald
Chair of the Department
Genetics of Microorganisms
and Speaker of the DFG-
GRK1870

Prof. Dr. rer. nat. Dr. med. vet. h. c.
Thomas C. Mettenleiter
President of FLI, Insel Riems

Prof. Dr. med.
Barbara M. Bröker

University Medicine
Greifswald
Chair of the Institute for
Immunology

RTG 1870 "Bacterial Respiratory Infections – Common and Specific Mechanisms of Pathogen Adaptation and Immune Defense"

CRC-TRR34 „Pathophysiology of Staphylococci in the Post-Genomic Era"

The Ernst-Moritz-Arndt-University Greifswald ("Alma Mater Gryphiswaldensis") was founded in 1456 and is considered to be one of the oldest academic institutions in Europe. Currently, over 10,000 students from all over the world benefit from a modern academic and research network in a time-honored environment. The members of the University´s community engage in interdisciplinary collaborations across faculties, universities and businesses, and aim for excellence in both research and teaching. Life sciences with a focus on cell and molecular biology, microbiology, infection biology, and biomathematics constitute one of the focal research areas. The University's scientific performance is likewise expressed by several multidisciplinary research programs funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) such as the Collaborative Research Centres (CRC) and the Research Training Groups (DFG-RTGs) for young scientists. The RTG 1870 "Bacterial Respiratory Infections – Common and Specific Mechanisms of Pathogen Adaptation and Immune Defense" investigates the adaptation and pathogenesis mechanisms of three major human respiratory bacterial pathogens (S. pneumoniae, S. aureus, and B. pseudomallei). In 15 different projects young researchers are given the chance to expand their methodological and social skills as well as to become integrated into the international scientific community. In the Transregional CRC 34 (CRC-TRR34) the Universities of Greifswald, Münster, Tübingen and Würzburg combine their expertise to study the "Pathogenesis of Staphylococci in the Post-Genomic Era". More than 50 researchers from a broad range of disciplines collaborate in around 20 projects funded by DFG for up to 12 years. To optimally support the early career researchers and facilitate networking between the research consortia, the CRC-TRR34 and the RTG 1870 closely collaborate with each other and interact with the University's Graduate Academy.

Friedrich-Loeffler-Institut
Federal Research Institute for Animal Health

The Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (FLI), addresses farm animal health and welfare and protection of humans from infections which can be transmitted between animals and humans. This includes the prevention, diagnostics and control of animal diseases, the improvement of animal welfare and animal nutrition as well as the preservation and use of farm animal genetic resources. The FLI is an independent higher federal authority under the auspices of the German Federal Ministry of Food and Agriculture (BMEL); central tasks are defined in the Animal Health Act. Furthermore, it is the national reference laboratory for all infectious diseases of animals including zoonoses, which are notifiable or reportable. At the international level, it fulfils numerous functions within the World Organization for Animal Health (OIE), the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). Today, 850 employees conduct research in 11 specialized institutes at five sites in Germany. They cooperate closely on individual research questions and collaborate with national and international research institutions in numerous projects. In addition to cooperation projects with various universities and research institutions, the FLI is involved in projects and missions of international organizations such as the OIE, the European Food Safety Authority (EFSA), the WHO and the FAO. Within the framework of scientific political consulting, the FLI draws up expert opinions and position statements. As a scientific research institution, the FLI acts as a consultant for political decision-makers at federal and EU level. The headquarters are located on Insel Riems, Greifswald. For research work on Insel Riems, laboratories and animal houses up to the highest biosafety level 4 are available. The bacteriologist and virologist Friedrich Loeffler founded the FLI at this site in 1910 – hence it is the oldest virus research institute in the world.

KoInfekt
"Elucidating Pathomechanisms of Bacterial-Viral Co-Infections with New Biomedical Models"

Bacto-viral co-infections are mixed infections where bacterial and viral pathogens are present simultaneously in the host and cause severe courses of disease. Humans and animals can be equally affected. Particularly co-infections of Influenza A viruses and bacteria causing severe pneumonia lead to high death rates every year.

The research programme KoInfekt, a newly established Mecklenburg-Pomerania Excellence Initiative project funded by the ESF, will investigate co-infections of Influenza A viruses with the most important causative bacterial agents of secondary infections, Streptococcus pneumoniae, Streptococcus suis, Staphylococcus aureus, and Streptococcus pyogenes.

Within 14 different projects, more than 40 scientists, including 17 doctoral researchers, aim to gain insights into pathogen-host interactions, the course of disease and induced host immune responses in order to develop novel strategies for control and prevention. In addition to mouse models, it is intended to establish pigs as biomedical models for co-infections. The genetic and physiological similarity between humans and pigs is high and, therefore, they might serve as suitable natural models for human infections. The Federal Research Institute for Animal Health on Insel Riems in the Greifswald area will contribute its expertise in the field of large animal infection research and virology. Research at the University of Greifswald will focus on small animal infection research, microbiology, immunology, and global OMICS analysis in cooperation with other KoInfekt partners at the University Medicines Greifswald and Rostock.

The results of KoInfekt will serve as basis for the development of more detailed prognoses for the severity of disease, establishment of molecular biomarkers, and improvement of clinical application.

Content

Program

Wednesday, November 1st, 2017

13:00 **Welcome**
Sven Hammerschmidt (Greifswald, Germany)
Barbara M. Bröker (Greifswald, Germany)
Thomas C. Mettenleiter (Insel Riems, Germany)

Topic 1: Virulence Strategies and Co-Infection

13:15 **Co-infections by respiratory pathogens in humans**
Sylvie van der Werf (Paris, France)

13:45 A glycine riboswitch controls expression of a sodium:alanine symporter family protein gene in Streptococcus pyogenes
Nadja Patenge (Rostock, Germany)

14:00 The influence of the HD-protein and the small RNA MOSES10 on the virulence of Streptococcus pyogenes
Mirijam Schäfer (Rostock, Germany)

14:15 How Staphylococcus aureus takes advantage of the non-polarized state of lung epithelial cells
Laura M. Palma Medina (Groningen, the Netherlands)

14:30 Regulation of fitness and colonization factors by the pneumococcal two-component regulatory system 08
Alejandro Gómez-Mejia (Greifswald, Germany)

14:45 **Experimental human co-infection with live attenuated influenza virus and pneumococcus**
Daniela M. Ferreira (Liverpool, United Kingdom)

15:15 Coffee break

Topic 2: Infection Immunology

15:45 **Streptococcus pneumoniae interactions with host immune responses**
Jeremy Brown (London, United Kingdom)

16:15 Mimicking in vivo growth conditions of Streptococcus pneumoniae
Lucille van Beek (Nijmegen, the Netherlands)

16:30 Biofilm in group A streptococcal necrotizing skin and soft tissue infections
Nikolai Siemens (Greifswald, Germany)

16:45 **Novel cellular regulators of inflammation in tuberculosis**
Anca Dorhoi (Insel Riems, Germany)

Program

17:15 The local and systemic NK cell response following respiratory influenza A virus infection depends on TLR7-signalling
Sabine Stegemann-Koniszewski (Otto-von-Guericke University, Magdeburg, Germany)

17:30 **Finding its intracellular niche - Chlamydia pneumoniae hides in the recycling system**
Johannes Hegemann (Duesseldorf, Germany)

19:00 Welcome Reception

Thursday, November 2nd, 2017

Topic 2: Infection Immunology

08:30 **Age and inflammation predispose to Streptococcus pneumoniae infection**
Dawn Bowdish (Hamilton, ON, Canada)

09:00 S. aureus serine proteases are inducers of airway allergies
Maria Nordengrün (Greifswald, Germany)

09:15 Drugs altering phagocytosis influence the survival of S. aureus
Vincent Péton (Greifswald, Germany)

09:30 Differential antibody response to the bacterial lipases Sal1 and Sal2 sheds light on the in vivo behavior of Staphylococcus aureus
Johannes Dick (Greifswald, Germany)

09:45 Targeting the anti-staphylococcal antibody response using a bead-based array approach
Tanja C. Meyer (Greifswald, Germany)

10:00 **Modelling staphylococcal pneumonia in a human 3D lung tissue model system**
Anna Norrby-Teglund (Stockholm, Sweden)

10:30 Coffee break

Program

Topic 3: Virulence Strategies

11:00 **Human respiratory syncytial virus (RSV) in the airway epithelium - ways to move around while staying under the radar of the adaptive immune response**
Ursula J. Buchholz (Bethesda, MD, USA)

11:30 Transgene expression in the genome of Middle East Respiratory Syndrome Coronavirus based on a novel reverse genetics system utilizing Red-mediated recombination cloning
Doreen Muth (Berlin, Germany)

11:45 **Human coronavirus-encoded proteins involved in de novo and primer-dependent RNA synthesis and replication fidelity**
John Ziebuhr (Giessen, Germany)

12:15 Genetic diversification of respiratory syncytial virus amongst hospitalized children in Heidelberg/Germany between 2014 and 2017
Clara Marie Ihling (Heidelberg, Germany)

12:30 Lunch

Topic 4: OMICS & Infection

13:30 In vivo proteome analysis of Streptococcus pneumoniae during CSF infections deciphers mechanisms of adaptation
Sven Hammerschmidt (Greifswald, Germany)

14:00 Protein arginine phosphorylation in Staphylococcus aureus
Sabryna Junker (Greifswald, Germany)

14:15 The missing links between staphylococcal epidemiology and intracellular survival – a quest for new diagnostic features
Solomon Mekonnen (Groningen, the Netherlands)

14:30 Inhibition of the transcription termination factor Rho by bicyclomycin affects expression of SaeSR-dependent virulence factor genes in Staphylococcus aureus
Anna Nagel (Greifswald, Germany)

14:45 Identification of the Lipoteichoic Acid Ligase in Streptococcus pneumoniae
Nicolas Gisch (Borstel, Germany)

15:00 **Metabolism meets virulence: Isotopologue profiling of intracellular pathogens and their host cells**
Wolfgang Eisenreich (Munich, Germany)

15:30 Poster Session

17:30 Departure to Conference Dinner at the Darwineum, Rostock Zoo (Bus Shuttle)

Program

Friday, November 3rd, 2017

Topic 5: Therapeutics and Vaccines

08:30 **Virus-induced transition from pneumococcal biofilm colonization to infection provides leads to novel vaccine strategies**
Anders P. Håkansson (Lund, Sweden)

09:00 Novel Double-Attenuated Influenza A Live Vaccines in Swine
Svenja Mamerow (Insel Riems, Germany)

09:15 Visualization of Haemophilus influenzae sialic acid theft from primary human bronchial epithelial cells and selective inhibition with sialic acid-based inhibitor abrogates serum resistance
Jeroen D. Langereis (Nijmegen, the Netherlands)

09:30 Intranasal vaccination with lipoproteins confers protection against pneumococcal colonization
Franziska Voß (Greifswald, Germany)

09:45 Antimicrobial activity in sputum from Intensive Care Unit (ICU) patients
Jolien Seinen (Greifswald, Germany)

10:00 **Therapeutic perspectives in pneumococcal pneumonia: From molecule to treatment**
Martin Witzenrath (Berlin, Germany)

10:30 Coffee break

Topic 6: Zoonoses

11:00 **The ecology of MERS-CoV: From host reservoir to disease**
Vincent Munster (Hamilton, MT, USA)

11:30 Virulence determinants of recent German avian influenza isolates subtype H7N7 in different host species
David Scheibner (Insel Riems, Germany)

11:45 Pathogenesis and transmission of the novel highly pathogenic avian influenza H5N8 2016 virus in ferrets and mice
Donata Hoffmann (Insel Riems, Germany)

12:00 Farewell
Sven Hammerschmidt (Greifswald, Germany)
Barbara M. Bröker (Greifswald, Germany)
Thomas C. Mettenleiter (Insel Riems, Germany)

12:15 Flying Lunch

Experimental human co-infection with live attenuated influenza virus and pneumococcus

Ferreira, Daniela M.

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Background: Pneumococcus is the most common cause of secondary pneumonia during influenza pandemics. As the nose is the reservoir of the bacteria, increased carriage density drives increased transmission of bacteria in the community during flu season, which can contribute to the increased burden of pneumonia.
Aim and Methods: We have employed live attentuated influenza virus (LAIV), which replicates in the nose but not in the lung, to elucidate how flu co-infection alters the mucosal immune responses that control pneumococcal carriage. Healthy adult volunteers received either nasal LAIV or nasal placebo three days before inoculation with pneumococcus. Carriage acquisition and density were assessed up to day 29. Nasal fluid and nasal cells were obtained longitudinally and bronchoalveolar lavages (BAL) were collected 21-184 days post inoculation.
Results: Antecedent LAIV caused a delayed clearance of pneumococcus following inoculation. Volunteers who received LAIV had transient increased acquisition and density of pneumococcus compared to controls. Carriage led to an early recruitment of monocytes to the nasal mucosa as well as increased pneumococcal specific responses. These responses were impaired by LAIV co-infection. Following co-infection, carriers had upregulated expression of pro-inflammatory genes and levels of pro-inflammatory cytokines/chemokines in the nasal fluid.
Conclusions: Increased pro-inflammatory responses following nasal flu infection predisposes to carriage acquisition and increased carriage density. LAIV impairs nasal monocyte response to pneumococcal carriage which could affect control of colonization density. LAIV vaccination also impairs the establishment of pneumococcus-specific immune responses in the nasopharynx.

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Regulation of fitness and colonization factors by the pneumococcal two-component regulatory system 08

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A global screening for potential regulation targets of the pneumococcal TCS08 was applied to D39 and TIGR4 isogenic mutants deficient in RR08, HK08 and both components of the TCS08 (Δrr08, Δhk08 and Δtcs08). Growth experiments in chemically defined medium (CDM) along with transcriptomics and functional assays unveiled changes in the gene expression of the arginine deaminase system (ADS), neuraminidases, and pilus type 1 (PI-1). The ADS is important for the arginine metabolisms, while neuraminidases and PI-1 are implicated in pneumococcal colonization. Furthermore, an extended adaptation phase for some of the mutants was observed. Nevertheless, the observed effects varied dependent on the genetic background. Results were confirmed by immunoblot analysis and flow cytometry using an array of antibodies recognizing specifically pneumococcal surface proteins, regulators or intracellular proteins. The results confirmed transcriptome analysis and importantly, indicated a substantially increased amount of PavB at a posttranscriptional level in the HK08 mutant in D39 and TIGR4. Strikingly, electromobility shift assays (EMSA) conducted with purified RR08 and a pavB promoter DNA-fragment illustrated binding of non- and phosphorylated RR08. Mouse infection models revealed an increased pathogenicity for the Δhk08 mutant in TIGR4 and a reduced virulence for the Δtcs08 mutants in D39. In conclusion, these data points on the regulation of pneumococcal colonization and fitness factors as a role for the TCS08 in S. pneumoniae.

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How Staphylococcus aureus takes advantage of the non-polarized state of lung epithelial cells

Palma Medina, Laura Marcela[1,2]; Hildebrandt, Petra[1]; Michalik, Stephan[1]; Gesell Salazar, Manuela[1]; Pförtner, Henrike[1]; van Dijl, Jan Maarten[2]; Völker, Uwe[1]

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The bronchial epithelium is the primary barrier protecting human lungs against infection. Upon injury, epithelial cell layers will regenerate but, initially, they are not polarized and susceptible to bacterial infections. This study aimed at understanding how bacterial pathogens take advantage of the non-polarized state of lung epithelial cells. Notably, the human pathogen Staphylococcus aureus is notorious for causing severe respiratory infections, especially necrotizing pneumonia. Therefore, we established an in vitro assay for S. aureus internalization by polarized or non-polarized human bronchial epithelial cells. At different time points post-infection, internalized bacteria were quantified by flow cytometry, and cellular adaptations in bacteria and epithelial cells were assessed by quantitative proteomics. Intracellular multiplication of S. aureus was observed in non-polarized cells, inducing apoptosis and membrane destruction. Conversely, bacteria did not proliferate in polarized epithelial cells which, accordingly, showed high survival rates. Altogether, 1361 bacterial proteins were quantified by 'data-independent analysis'. Significant quantitative differences in at least 20% of the proteome reflected the main adaptive responses to the conditions within polarized or non-polarized cells. Consistent with high bacterial replication rates within the non-polarized epithelium, many changes related to metabolism. Noticeable polarization state-dependent differences also included proteins with functions in cell signaling, stress management, and virulence. Major adaptations were already detectable 2.5 h post-internalization, indicating that early stages of infection could be decisive for the course of pulmonary disease. We consider these observations important, because a detailed understanding of pathogen adaptations to the regenerating epithelium is critically needed to protect patients with pulmonary diseases.

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A glycine riboswitch controls expression of a sodium: alanine symporter family protein gene in Streptococcus pyogenes

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Bacterial riboswitches are non-coding RNA elements controlling gene expression in response to environmental signals. In this study, we investigated gene expression regulation by a putative glycine riboswitch located in the 5'-UTR of a sodium:alanine symporter family protein (SAF) gene in in the group A Streptococcus pyogenes M49 591. The sequence of the cis-regulatory element is homologous to a riboswitch in Bacillus subtilis, which induces production of glycine degrading enzymes in the presence of glycine. We asked, whether riboswitch-mediated gene regulation in S. pyogenes is controlled by a similar mechanism. Glycine-dependent gene expression was studied using a luciferase (LUC) reporter gene system. Maximal reporter gene expression was observed in the absence of glycine and in the presence of low glycine concentrations. At high concentrations of glycine (≥ 1 mM), LUC expression was repressed. Alanine and serine did not influence LUC activity. Expression of the SAF gene and the downstream putative cation efflux protein gene in WT bacteria was investigated by RT-qPCR transcript analyses. In the presence of glycine (≥ 1 mM), expression of both genes was downregulated. Northern blot analyses revealed premature transcription termination in the presence of high glycine concentrations. Full-length transcripts were synthesized in the absence of glycine or at a low glycine concentration (0.1 mM). Furthermore, stability of the SAF gene transcript was drastically reduced in the presence of glycine. SAF are known to be responsible for alanine and glycine import. Since glycine is essential for GAS growth, the glycine riboswitch is a potential therapeutic target.

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The influence of the HD-protein and the small RNA MOSES10 on the virulence of Streptococcus pyogenes

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Streptococcus pyogenes is a Gram-positive strictly human pathogen, which causes local skin and throat infections. Additionally, it is responsible for severe invasive diseases and autoimmune responses like acute rheumatic fever. The expression of streptococcal genes is regulated by independent response regulators and two-component signal transduction systems. Studies on small regulatory RNAs show their potential role in the expression control of bacterial virulence-genes. Preliminary results suggested that the small RNA candidate gene moses10 and the down-stream located HD-protein gene influence each other. In this project, the influence of the HD-protein and the small RNA MOSES10 on the virulence of S. pyogenes was studied. Proteins containing HD domains belong to the super family of metal-dependent phosphatases and play a role in nucleic acid metabolism and signal transduction. In Enterococcus faekalis, the EF1143 protein, containing an HD domain, showed a nuclease-activity and a triphosphohydrolase-(dNTPase-) activity.
Recombinant HD-protein, fused to a Strep-taq® for purification, was produced in E. coli BL21 DE3. For enzymatic characterization, nuclease assays and a two-step colorimetric phosphohydrolase assay were performed. The influence of the HD-protein and MOSES10 on virulence was studied using a Galleria mellonella infection model with HD-protein gene and moses10 deletion and complementation strains.
Preferred substrates for the nuclease activity of the HD-protein were ssDNA and linear dsDNA. The four canonical dNTPs and TTP could be identified as suitable substrates for the phosphohydrolase-activity. Galleria mellonella survival rates were increased following infection with the deletion strains compared to WT, suggesting an influence of both genes on S. pyogenes virulence.

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Co-infections by respiratory pathogens in humans

van der Werf, Sylvie[1,2]

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Acute respiratory infection (ARI) is a leading cause of morbidity and mortality worldwide, particularly in children but also in older adults. The main viral pathogens associated with ARI include influenza viruses, respiratory syncytial virus (RSV), human metapneumovirus (hMPV) and rhinoviruses. Co-infections with multiple respiratory viruses are frequently observed as well as simultaneous or sequential infections with bacteria. Although respiratory infections are often relatively mild and confined to the upper respiratory tract, progression to the lower respiratory tract may result in severe disease requiring hospitalization. However, unraveling the clinical significance of respiratory pathogens co-infections is a complex issue. We will review the epidemiology of respiratory infections and most common co-infections with focus on influenza virus and RSV in humans, summarize current evidence for the clinical significance of co-infections and implications for transmission, and discuss mechanisms of cooperative interactions between these respiratory pathogens and potential for treatment and prevention strategies.

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Age and inflammation predispose to Streptococcus pneumoniae infection

Bowdish, Dawn

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Streptococcus pneumoniae colonization is a pre-requisite to infection. Older adults have a low incidence of pneumococcal colonization but extremely high rates of infection. How do we explain this paradox? Monocytes and macrophages are essential for control of colonization but age-related changes in their function result in less effective control of colonization. Specifically, age-related inflammation alters monocyte development and age-related changes in phenotype and function that contribute to susceptibility to disease. Using an aged mouse model of pneumococcal infection we have discovered that monocytes derived from old (18-22 mo) mice home more readily to sites of infection than young mice (10-14 wk). These monocytes are immature, however, an as a result, they produce more inflammatory cytokines and are less able to bind bacteria. Macrophages derived from them are hyper-inflammatory and less able to kill internalized bacteria. By studying the role of age-associated inflammation in monocyte and macrophage development in both humans and mice, we propose a model in which age and chronic inflammation subtly alter the inflammatory tone of the host which in turn alters monocyte and macrophage development and propose that novel therapeutic targets need to be tailored to the age of the host.

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Streptococcus pneumoniae interactions with host immune responses

Brown, Jeremy

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Streptococcus pneumoniae is the commonest bacterial cause of pneumonia, often associated with septicaemia and a high case fatality rate. S. pneumoniae is particularly common in the very young and the elderly, with exponential increases in incidence after the age of 65 years. The reasons for the high susceptibility of the elderly to S. pneumoniae pneumonia are not clear, but are likely to be partially related to immunosenescence. As well as being a common pathogen, S. pneumoniae is a very common commensal of the nasopharynx. Recent evidence has demonstrated that these colonisation events induce adaptive immunity that helps protect the host against invasive infection. Work in Professor Brown's laboratory as investigated the mechanisms of this naturally acquired adaptive immune response. The main finding has been that unlike existing vaccines, naturally acquired adaptive immunity to S. pneumoniae targets protein antigens including well known surface proteins. These responses seem to be largely conserved in different populations, although with some variation between individuals in these populations. In this presentation, Prof Brown will discuss these data and their consequences for vaccine and other preventative strategies.

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Differential antibody response to the bacterial lipases Sal1 and Sal2 sheds light on the in vivo behavior of Staphylococcus aureus

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Staphylococcus aureus is a common commensal but can also cause severe infections. This ambiguity can, at least partly, be explained by the multifaceted interactions between the bacterium and the immune system. To identify immunodominant bacterial proteins, the research group examined sera from colonized subjects and bacteraemia patients for antibodies against the proteins expressed by their respective strain. One protein stood out as an irregularity: S. aureus lipase 1 (Sal1), a secreted lipolytic enzyme. Sal1 is highly abundant and very conserved, suggesting that it is an important enzyme in S. aureus' virulence or general fitness. Unexpectedly, humans rarely produce considerable levels of anti-Sal1-IgG or IgM. For a protein thought to be regularly expressed, such an antibody gap is highly unusual. A second lipase (Sal2) has high structural homology but elicits a regular antibody response, making the observed gap for Sal1 all the more intriguing.
We characterized the cellular response to Sal1 in vitro and found normal levels of T cells with a typical Th1/Th17 phenotype in humans, indicative of a regular T cell memory response. We observed no detrimental effect of Sal1 on B cells or professional phagocytes.
We therefore propose that in vivo Sal1 is exclusively expressed under conditions rendering an IgG response impossible, for example within the phagosome of professional phagocytes, within biofilms inaccessible to B cells, or on the skin surface. This hypothesis is backed by several transcriptomic and proteomic studies as well as first data indicating the importance of S. aureus lipases in intracellular survival.

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Novel cellular regulators of inflammation in tuberculosis

Dorhoi, Anca

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Myeloid cells are critical for pathogenesis of tuberculosis (TB), a chronic pulmonary infection causing morbidity and mortality worldwide. Macrophages represent bona fide host cells for Mycobacterium tuberculosis (Mtb) which is the etiologic agent of TB. Along with other phagocytes, including dendritic cells and neutrophils, macrophages orchestrate inflammation in this disease. Recently, we have identified myeloid-derived suppressor cells (MDSC), a subset of phagocytes restricting lymphocyte functionality, at the site of infection in murine TB. Interaction of human monocytic MDSC with mycobacteria, as well as their effect on stability of granulomas will be discussed in view of recent experimental evidence. Versatility of myeloid cell subsets and their impact on pulmonary inflammation will be further emphasized by findings from experimental murine TB. Our studies unveil that in situ cross talk between lung-residing phagocytes and hemostasis effectors promotes primary progressive TB. Targeting newly identified checkpoints of inflammation represents a promising approach for the design of host-directed therapies for TB.

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Finding its intracellular niche - Chlamydia pneumoniae hides in the recycling system

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Chlamydia pneumoniae causes acute and chronic respiratory tract diseases. Binding of the chlamydial invasin Pmp21 to the epidermal growth factor receptor (EGFR) results in receptor activation. The internalized bacteria remain associated with activated EGFR throughout infection. Thus, C. pneumoniae must somehow intervene in EGFR-mediated events so as to avoid EGFR-triggered degradation or rerouting back to the plasma membrane. The fate of every endocytic process is decided by the early endosome (EE) or sorting endosome (SE) and orchestrated by the presence of various small Rab GTPases.

We now show that the early C. pneumoniae endosome harbors phosphatidylinositol 3-phosphate (PI3P) membrane identity. This early chlamydial inclusion acquires the early endosomal Rab GTPases Rab4, Rab5, Rab7, as well as the two recycling-specific Rabs Rab11 and Rab14. While Rab5, Rab11 and Rab14 are retained in the vesicular membrane, Rab4 and Rab7 disappear. Loss of Rab7 enables the C. pneumoniae inclusion to escape lysosomal degradation. Loss of Rab4 and retention of Rab11/ Rab14 designates the inclusion as a slowly recycling endosome – that is protected from degradation. The Rab11/ Rab14 adaptor protein Rab11-Fip2 (Fip2) is also recruited to the nascent inclusion. Fip2 knockdown demonstrates that the protein is essential for internalization and infection. Moreover, Rab11 and Fip2 recruit the unconventional actin motor protein myosin Vb which regulate the relocation of the nascent inclusion to the perinuclear region, its final destination.

Thus, construction of the C. pneumoniae intracellular niche is key to survival of the bacteria within the host cell (Mölleken + Hegemann, PLOS Pathogens, 2017).

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Targeting the anti-staphylococcal antibody response using a bead-based array approach

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Around 30 % of the human population is asymptomatically colonized with Staphylococcus aureus. However, this commensal can also be a pathogen causing a wide range of sometimes live-threatening conditions. Both carriers and non-carriers display a broad range of anti-staphylococcal antibodies with pronounced inter-individual variations in antibody specificities and titers [Verkaik 2009, JInfectDis]. Studies of bacteremia patients indicate that these antibodies might have protective potential [Stentzel 2016, JProteomics]. However, the factors shaping the antibody repertoire have only partially been explored. To gain a better understanding of determinants of the human antibody repertoire, the humoral response to a total of 148 heterologously expressed or chemically synthesized staphylococcal antigens was recorded in pooled serum or plasma samples of different patient groups as well as in healthy individuals (carriers and non-carriers). The patients were either infected with S. aureus or known to exhibit high colonization rates, caused, for example, by an impaired skin barrier function or by dysregulation of the immune system (atopic dermatitis, asthma, cystic fibrosis, epidermolysis bullosa, S. aureus bacteremia, requirement for dialysis). Serial dilutions of all serum pools were incubated with xMAP®-beads (Luminex®) presenting the antigens in a multiplexed assay. Bound antigen-specific serum antibodies were quantified using detection antibodies against total IgG, IgA and IgG4. The complex raw dataset was quantitatively evaluated with a newly developed analysis pipeline. This data mining strategy as well as the complex variations in the antibody profiles of the three analyzed immune globulin classes between the included groups of patients and healthy carriers/non-carries will be presented.

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S. aureus serine proteases are inducers of airway allergies

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According to the hygiene hypothesis, childhood infections as well as early exposure to microbial diversity protect from allergy. However, there is increasing evidence that besides commensal and invasive behavior S. aureus may also drive allergic reactions. In patients suffering from allergic (Th2 biased) disorders, e.g. asthma and nasal polyposis, S. aureus colonization is much more frequent. However, the driving allergens of S. aureus remained elusive.
By imunoblotting of S. aureus extracellular proteins we could identify the serine protease-like proteins (Spls) A-F as major IgG4-binding proteins. IgG4 served as a surrogate marker for IgE, since production of IgG4 and IgE are both initiated by a similar Th2 cytokine profile. The S. aureus Spls A-F are extracellular proteases of so far unknown function.
We observed increased Spl-specific serum IgE titers in asthma patients. Following stimulation with Spls, memory T cells of healthy donors secreted Th2 cytokines. In contrast, Th1/Th17 cytokines were of low concentration or absent. In mice, intra-tracheal application of SplD without adjuvant induced allergic lung inflammation, including infiltration of inflammatory cells and induction of SplD-specific serum IgE. This identifies SplD as a triggering allergen of S. aureus. To elucidate the underlying mechanism, we blocked the downstream effects of IL-33 by co-administration of sST2, the soluble IL-33 receptor. sST2 treatment counteracted the SplD-mediated allergic lung inflammation, indicating that SplD causes a Th2 response in an IL-33 dependent manner.
In summary, we identified Spls as triggering allergens by S. aureus, opening prospects for diagnosis and causal therapy of asthma.

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Modelling Staphylococcal pneumonia in a human 3D lung tissue model system

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Staphylococcus aureus necrotizing pneumonia is recognized as a toxin-mediated disease, but yet the tissue destructive events remain elusive partly due to lack of mechanistic studies in human lung tissue. In this study, a 3D-tissue model composed of human lung epithelial cells and fibroblasts was used to delineate the role of specific staphylococcal exotoxins in tissue pathology. The models were exposed to the mixture of exotoxins produced by S. aureus strains isolated from patients with varying severity of lung infection, namely necrotizing pneumonia (NP) or lung empyema (LE), or to purified toxins. The NP strains secreted high levels of α-toxin and PVL, and triggered high cytotoxicity, inflammation, necrosis and loss of E-cadherin in the lung epithelium. In contrast, the LE strain produced moderate levels of PVL, but no α-toxin, and triggered limited tissue damage. α-toxin had a direct damaging effect on the epithelium, as verified by toxin-deficient mutants and pure α-toxin. PVL contributed to lung injury through the lysis of neutrophils, and a combination of α-toxin and PVL resulted in the most severe tissue injury. The study also revealed an α-toxin dependent modulation of chemokine responses translating into increased chemotactic activity. Association between toxin levels, cytotoxicity and clinical outcome was confirmed in a larger cohort of pneumonia isolates. This study introduces a novel model system for studies of staphylococcal pneumonia in a human tissue setting, and the results revealed that a combination and the levels of α-toxin and PVL correlate with tissue pathology and clinical outcome associated with pneumonia.

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Drugs altering phagocytosis influence the survival of S. aureus

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Dendritic cells (DCs) are important professional phagocytes and antigen-presenting cells connecting the innate and the adaptive immune system. DCs sample surrounding antigens via endocytosis and present processed peptide fragments. However some pathogens, like Staphylococcus aureus, can survive to this process. Here, we explore the impact of chloroquine, bafilomycin-A1, cytochalasin-D or wortmannin, which interfere with phagocytosis or autophagy mechanisms, on the intracellular fate of S. aureus.

To check if genetic background could influence of the drugs efficiency, human or murine S. aureus strains CC88 and CC49 were modified to express GFP in an infectious context. Dendritic cells from the JAWSII cell line were infected with these strains after incubation with the drugs. Internalized bacteria were numerated and observed with live fluorescence microscopy. Cytokines produced by the DCs were titrated during the course of infection.

By live fluorescence microscopy, we observed that S. aureus can persist inside the dendritic cells after phagocytosis, but can also escape and invade the whole cytoplasm, leading to the host cell death. Surprisingly, the chloroquin and the bafilomycin-A1, two drugs that prevent the phagosome acidification, led to a decreased number of intracellular S. aureus. It appears that the genetics of the strain influences the intracellular survival but it has little influence on the drug effect.

These results suggest the drugs can modify the intracellular survival of S. aureus in DCs by increasing the lysis in the phago(lyso)somes, but once the bacteria escaped from these compartments they can kill their host cell and infect the surrounding cells.

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Biofilm in group A streptococcal necrotizing skin and soft tissue infections

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Background: Necrotizing fasciitis caused by group A streptococcus (GAS) are life-threatening, rapidly progressing infections, which often requires extensive and repeated surgical procedures, suggesting a problem with bacterial persistence. Until now, no evidence of biofilm in necrotizing fasciitis was shown. Here, we report the emergence of biofilm in GAS-mediated necrotizing fasciitis.
Methods: Biofilms were identified in tissue biopsies from GAS NSTI patients, as well as a human 3D skin tissue infected with clinical strains by as light, confocal, and electron microscopy analyses. Bacterial and host factors were assessed by immunostainings and microscopy analyses, ELISA assays, and/or qRT-PCR.
Findings: After a report of "rich layer biofilm" in one patient by a surgeon, patients biopsies collected from the affected deep tissue site were analyzed and extensive multiple areas of biofilm were identified. Biopsies associated with biofilm formation were characterized by a pronounced host response in terms of elevated levels of CXCL8, resistin, and neutrophil influx. In vitro infections of engineered unique human tissue with clinical GAS emm1, emm3, and emm49 NSTI strains identified multilayered fibrous biofilm structures.
Interpretation: The novel finding of biofilm formation in NSTIs is of considerable clinical relevance as it hampers efficient diagnostics and is associated with antibiotic resistance. That emphasizes the urgent need in re-consideration of the treatment protocols.

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The local and systemic NK cell response following respiratory influenza A virus infection depends on TLR7-signalling

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Influenza A virus (IAV) remains a serious burden for human health and a full understanding of its pathogenesis is prerequisite for the advancement of anti-viral measures. The innate immune system senses IAV through different pathogen-recognition receptors (PRR) including Toll-like receptor 7 (TLR7) and in the early course of IAV infection natural killer (NK) cell responses develop. Whereas the decisive role for TLR7 in NK cell activation through therapeutic immuno-stimulatory RNAs has been described, this PRRs contribution to the NK cell response following IAV infection has not been addressed. We have previously described an attenuated interferon (IFN)-γ response in the lungs of IAV infected TLR7-deficient mice and have now analyzed their activation of lung NK cells. Indeed, lung NK cell CD69 expression and IFN-γ production following sublethal IAV infection were significantly attenuated in TLR7-deficient compared to wild-type hosts. Strikingly, respiratory IAV infection also primed splenic NK cells for IFN-γ production, degranulation and target cell lysis, which was fully dependent on TLR7. While lung interleukin-12 was unchanged, type I interferon (IFN I) levels were significantly reduced in TLR7ko mice early following infection, displaying a potential upstream mechanism of the defects in NK cell activation. Interestingly, the attenuated IFN-γ and IFN I response detected in TLR7ko mice correlate well with their benefit in secondary bacterial infection we have previously observed. Taken together, our data for the first time demonstrate the specific contribution of TLR7-signalling to NK cell activation following respiratory IAV infection despite the presence of redundant innate recognition pathways.

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Mimicking in vivo growth conditions of Streptococcus pneumoniae

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Streptococcus pneumoniae is a bacterial pathogen and a major cause of morbidity worldwide, ranging from otitis media and sinusitis to more severe and invasive diseases such as pneumonia, meningitis and sepsis, associated with high mortality rates. Conjugate polysaccharide vaccines have been developed to prevent pneumococcal infections, although only 13 of the more than 90 different serotypes are covered, leading to vaccine escape as a consequence of serotype replacement. In addition, these vaccines are unaffordable for a large number of developing countries, which hampers the reduction of pneumococcal disease. Vaccines consisting of broadly protective protein-based antigens is one of the most promising approaches to prevent pneumococcal infections.
To identify candidate protein antigens, we aim to mimic the in vivo growth conditions of S. pneumoniae. A prerequisite for infection by S. pneumoniae is nasopharyngeal colonization, a generally nutrient-poor environment. Especially metal ion homeostasis is important for survival, hence persistent colonization of S. pneumoniae. However, the nasopharyngeal metal levels are unknown. We collected nasal fluid of healthy adults and measured the concentration of metal ions by induction-coupled plasma mass spectrometry. Trace amounts of manganese and cobalt were determined (<1 µM), whereas copper and zinc levels were at least 10-fold higher. Using this data a chemically defined medium was designed that mimics the metal ion levels in human nasal fluid. Cell wall fractions from pneumococci grown under these in vivo-mimicking conditions will be compared to standard in vitro conditions, to characterize potential antigens by mass spectrometry.

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Human respiratory syncytial virus (RSV) in the airway epithelium - ways to move around while staying under the radar of the adaptive immune response

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Respiratory syncytial virus (RSV) is the most important viral agent of severe pediatric respiratory disease. While re-infection by Influenza A virus (IAV) depends on antigenic change, RSV re-infects throughout life without need of significant antigenic change. Dendritic cells (DC) are critical for directing the adaptive response to RSV. We found that, compared to IAV, RSV only weakly stimulates DC in vitro, and only weakly upregulates CCR7, a DC chemokine receptor essential for migration of DC to lymphatic tissues. Suboptimal stimulation rather than viral inhibition seems to be responsible for the poor stimulation of DC by RSV. RSV preferentially replicates and effectively spreads in the superficial layer of airway epithelial cells. We found that the RSV NS2 protein promotes shedding of superficial airway epithelial cells. This NS2-induced airway epithelial cell shedding likely accelerates viral clearance, and possibly viral spread, but it also contributes to airway obstruction, identifying NS2 as an RSV pathogenicity factor. Also, RSV infection increases the motility of infected airway epithelial cells, and induces the formation of cell protrusions (filopodia) in infected cells. RSV-induced motility and filopodia formation are dependent on actin related protein 2 (ARP2), part of the ARP2/3 actin nucleation complex. Interestingly, the RSV induced filopodia seem to shuttle RSV to neighboring uninfected cells, contributing to viral spread. Thus, RSV has ways to efficiently spread in superficial epithelial airway cells, while keeping a low profile for the adaptive immune response. The low invasiveness and the efficient viral spread likely contribute to the ability to effectively re-infect.

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Genetic diversification of respiratory syncytial virus amongst hospitalized children in Heidelberg/Germany between 2014 and 2017

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Background: Respiratory syncytial virus (RSV) is the leading cause of hospitalization especially in young children with respiratory tract infections (RTI). The aim of this research project is to analyse the spread and diversification of RSV genotypes amongst hospitalized children in Heidelberg/Germany.
Methods: We prospectively analysed nasopharyngeal swabs (NPS) from hospitalized children (<18 years) who presented with acute RTI at the University Hospital Heidelberg/Germany during winter seasons 2014 to 2017. We performed rtPCR and RSV sequence analysis of the second variable region of the G-gene coding for the attachment glycoprotein. Clinical data was obtained using a standardized questionnaire.
Results: RSV was detected in 444/1078 samples (2014/15: n=111/235, 2015/16: n=104/325, 2016/17: n=229/518). Most RSV-positive children were below the age of two years (85.8 %) and had a lower RTI (78.6 %). Phylogenetic analysis of 369 isolates revealed that majority of RSV-A strains (n=208/210) belonged to the novel ON1 genotype containing a 72-nucleotide duplication, only one strain was genotype NA1 and GA5, respectively. Most RSV-B strains could be attributed to the BAIX genotype (n=156/159), two strains to genotype BAX and one to genotype BAIV, all containing a 60-nucleotide duplication. ON1 and BAIX strains could be subdivided into several clusters of new variants (ON1 n=12, BAIX n=9) which might eventually evolve into further (sub)genotypes.
Conclusion: Mapping the spread of novel genotypes using data from different seasons can reveal transmission dynamics and fitness of viral strains. Further surveillance of circulating genotypes in combination with corresponding clinical data is needed to understand their full implications.

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Transgene expression in the genome of Middle East Respiratory Syndrome Coronavirus based on a novel reverse genetics system utilizing Red-mediated recombination cloning

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Middle East respiratory syndrome coronavirus (MERS-CoV) is a high priority pathogen in pandemic preparedness research. Reverse genetics systems are a valuable tool to study viral replication and pathogenesis, design attenuated vaccines, and create defined viral assay systems for applications such as antiviral screening. Here we present a novel reverse genetics system for MERS-CoV that involves maintenance of the full-length viral genome as a cDNA copy inserted in a bacterial artificial chromosome amenable to manipulation by homologues recombination based on the bacteriophage λ Red recombination system. Based on a full-length infectious MERS-CoV cDNA clone, optimal genomic insertion sites and expression strategies for GFP were identified and used to generate a reporter MERS-CoV expressing GFP in addition to the complete set of viral proteins. GFP was genetically fused to the N-terminal part of protein 4a, from which it is released co-translationally via a self-cleaving porcine teschovirus 2A peptide. The resulting reporter virus achieved replication levels nearly identical to the wild-type virus (1x 105 p.f.u./ml and 3x 105 p.f.u./ml, respectively) and allowed to determine the 50 % inhibitory concentration for the known MERS-CoV inhibitor cyclosporine A based on fluorescence readout. The resulting value was 2.41 µM, which corresponds to values based on wild type virus. The here-presented reverse genetics system can be efficiently mutated by Red-mediated recombination. The GFP-expressing reporter virus contains the full set of MERS-CoV proteins and achieves wildtype replication level in cell culture.

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Human coronavirus-encoded proteins involved in de novo and primer-dependent RNA synthesis and replication fidelity

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Human coronaviruses are important respiratory pathogens associated with both upper and lower respiratory tract infections. Coronaviruses have very large RNA genomes, employ unique strategies in genome replication, and encode a number of enzymes that modulate host cell functions. Recently, we have been able to reconstitute complexes of coronavirus-encoded proteins that support primer-dependent and de novo RNA synthesis in vitro. The complexes were assembled from recombinant proteins produced in E. coli and then used to investigate the specific requirements for coronavirus RNA synthesis. We also obtained evidence to suggest role(s) for specific viral proteins in coronavirus RNA processing, such as 3' polyadenylation and RNA 5' cap 1 formation. Unlike other RNA viruses, coronaviruses and several related nidoviruses appear to have evolved special mechanisms to improve RNA replication fidelity, thereby keeping the error frequency of the viral polymerase below a postulated critical threshold. A possible key player in this process was suggested to be a coronavirus replicase gene-encoded 3'-to-5' exoribonuclease associated with nonstructural protein (nsp) 14. Characterization of a recombinant form of this protein revealed insight into the enzyme's substrate requirements and supports previous predictions on a possible involvement of nsp14 in proofreading activities of the coronavirus replication complex. Our studies suggest that, in the course of evolution, genome size expansion in coronaviruses and related nidoviruses to more than 30 kb gave rise to complex assemblies of highly specialized enzyme functions that (i) mediate individual steps of viral RNA synthesis and processing and (ii) affect host cell functions.

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Metabolism meets virulence: Isotopologue profiling of intracellular pathogens and their host cells

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Metabolic adaptation is a key feature of pathogenic bacteria during infection. As a result of this adaptation process, the metabolic pathways and fluxes of the pathogen, but also of their hosts are modulated to the benefit or disfavor for the partners. For this complex metabolic interplay, the term "pathometabolism" has been coined. There are now plenty of examples that intracellular pathogens including Legionella pneumophila, Chlamydia trachomatis, Listeria monocytogenes, and Mycobacterium tuberculosis specifically adapt their nutrient usages, the metabolic pathways and fluxes to the various environments encountered during their infection processes. On the other hand, the metabolic processes of the respective host cells also seem to be changed during infections, when the pathogens efficiently retrieve nutrients during their replication. A powerful method to analyze metabolic pathways and fluxes under complex conditions is based on 13C- or 15N-incorporation experiments with the respective pathogens in vitro or using infected host cells/organisms. Isotope enrichments and positional isotope distributions in multiple metabolic products from the bacteria and the hosts can be analyzed by MS and/or NMR providing detailed quantitative information about the pathometabolism of intracellular bacteria.

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Identification of the Lipoteichoic Acid Ligase in Streptococcus pneumoniae

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The teichoic acids of Streptococcus pneumoniae (pnTAs) are unique in several aspects. First, they are highly decorated with phosphorylcholine (P-Cho), which non-covalently anchors choline-binding proteins to the pneumococcal surface. Second, pneumococcal WTA (pnWTA) and LTA (pnLTA) chains have identical and chemically complex repeating unit structures produced in a common precursor synthesis pathway. Members of the LytR-Cps2A-Psr (LCP) protein family are suggested to have semi-redundant roles in capsule attachment and transfer of pnTA precursor chains to the peptidoglycan (PGN) to form the pnWTA. Additionally, an involvement in the transfer of the TA precursor chains to the glycolipid anchor to form the LTA was assumed.
In our study we show that pnLTA exhibits a different linkage configuration than pnWTA-PGN. We identified the enzyme which is necessary for the attachment of the pnTA precursor to the glycolipid anchor to form the pnLTA, which we name TacL for ´lipoteichoic acid ligase´. TacL mutants lacked pnLTA, whereas pnWTA was still present. Interestingly, tacL mutants grew normally in the laboratory and were phenotypically similar to the respective isogenic wild-type strains, but showed drastically reduced virulence in murine models of acute pneumonia and systemic infections.

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In vivo proteome analysis of Streptococcus pneumoniae during CSF infections deciphers mechanisms of adaptation

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Pathogenic bacteria encountering various host compartments during invasive infections have to adapt their physiology and virulence potential to changing host niche conditions. The dynamics of the proteome reflects the adaptation to a certain host niche. Here we have employed an in vivo proteomics-based approach to identify pneumococcal factors contributing to cerebrospinal fluid (CSF) infections. A comprehensive and qualitative mass spectrometry (MS) spectra library was generated enabling bacterial proteome analysis even in the presence of eukaryotic proteins. In vitro samples resulted in a spectra library of 7,597 unique peptides corresponding to 1,165 proteins. Approximately 200,000 pneumococci were recovered from the CSF of mice with pneumococcal meningitis by a dual filter extraction step. High sensitive MS including spectra to spectra comparison identified 685 proteins in the infection dose (control sample) and 249 proteins from CSF recovered pneumococci. The two-component regulatory system ComDE, important for pneumococcal competence, and the substrate-binding protein AliB of an ABC oligopeptide transport system were exclusively detected post infection. To demonstrate the crucial role of AliB and ComDE mutants deficient in AliB, ComDE or AliB/ComDE were generated and the in vivo effect analysed. In the meningitis model, AliB-, ComDE-, or AliB-ComDE-deficiency resulted in attenuated meningeal inflammation and disease course compared to that induced by the wild-type strain. We further characterized the expression profiles of the mutants by proteomics. Combined, the in vivo proteomics approach is a powerful tool to characterize protein dynamics of pathogens during host infections and identifies crucial players involved in virulence, fitness and gene regulation.

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Protein arginine phosphorylation in Staphylococcus aureus

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Reversible protein phosphorylation is one of the major mechanisms in the regulation of protein expression and protein activity, controlling central cellular functions of the important human pathogen Staphylococcus aureus. Therefore, investigation of the phosphoproteome will help to decipher molecular and cellular mechanisms that underlie pathogenesis and virulence. In addition to the well-known o-phosphorylations, the phosphorylation at arginine residues is likely to play an essential, but mostly still unknown role in Gram positive bacteria. Therefore we decided to study arginine phosphorylations in greater detail.
S. aureus COL possesses the protein PtpB, which was assumed to be an arginine phosphatase. The construction of the deletion mutant ΔptpB therefore aimed to increase the level of arginine phosphorylations, making them more accessible to proteomic analyses. Hence, we applied a gel-free method to analyze the changes in the phosphoproteome of the deletion mutant ΔptpB and the wild type, thereby focusing on arginine phosphorylations.
In order to enhance the number and reproducibility of identified phosphorylation sites at arginine residues, a subset of arginine phosphorylated peptides was chemically synthesized. The analysis of synthetic peptides and experimental data by spectral library based methods provides an additional tool (besides classical database search) to address the challenges of analyzing arginine phosphorylations. We provide a combined spectral library covering 84 % of the theoretically predicted proteome. 396 arginine phosphopeptides within the spectral library allowed the identification of 207 arginine phosphosites exclusively within the mutant. This identification of putative targets of PtpB allows further investigation of the physiological relevance of arginine phosphorylations.

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The missing links between staphylococcal epidemiology and intracellular survival – a quest for new diagnostic features

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Methicillin-resistant Staphylococcus aureus (MRSA) isolates represent a heterogeneous group of highly drug-resistant staphylococci. Two major MRSA classes are distinguished based on epidemiology, namely community-associated (CA) and hospital-associated (HA) MRSA. The distinction of both classes based on molecular traits is challenged by the high genomic plasticity of S. aureus. Here we sought to pinpoint global distinguishing features of CA- and HA-MRSA through a comparative genome, transcriptome and proteome analysis of S. aureus USA300 isolates. This is an important objective in the context of respiratory infections, because CA-MRSA is associated with necrotizing pneumonia. Comparative genome analysis suggests that the accessory genome is a distinctive feature of CA- and HA-MRSA isolates. Intriguingly, RNA sequencing revealed differential expression of 208 genes in the CA- and HA-MRSA isolates, relating to major virulence factors, stress responses and the histidine, purine, pyrimidine and fatty acid biosynthetic pathways. Importantly, expression of most of the virulence factors is correlated to differential regulation of the agr system. In addition, our exo-proteome analysis separated CA- and HA-MRSA isolates by two distinct extracellular cytoplasmic protein abundance clusters. Such extracellular cytoplasmic proteins were recently proposed to be involved in staphylococcal virulence. Importantly, the outcomes of the RNA sequencing and proteomics analyses are mirrored by differences in the ability of CA- and HA-MRSA to thrive and survive within human lung epithelial cells and neutrophils. Altogether, our findings thus connect differential epidemiological behavior of related MRSA isolates to distinguishing and potentially diagnostic molecular features..

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Inhibition of the transcription termination factor Rho by bicyclomycin affects expression of SaeSR-dependent virulence factor genes in Staphylococcus aureus

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In a comparative OMICs-study Staphylococcus aureus HG001, a derivative of strain NCTC 8325, and its isogenic Δrho mutant were analyzed using strand-specific tiling arrays [Mäder et al., 2016]. This analysis revealed a relatively low abundance of antisense RNAs in the S. aureus wild type. The transcription termination factor Rho plays a major role in suppressing antisense transcription in E. coli and B. subtilis, and indeed there is a remarkable overall increase in antisense transcription in the absence of Rho in S. aureus. Proteome analyses of cytoplasmic and secreted fractions comparing S. aureus HG001 wild type and the Δrho mutant showed significant differences in the abundance of several proteins, namely increased amounts of SaeSR-dependent virulence factors in the rho mutant.
Bicyclomycin (BCM) is an antibiotic, which specifically inhibits Rho and can be used for treatment of Gram-negative bacteria. Therefore, we sought to analyze if treatment of S. aureus wild type cells with BCM would result in the same effects on the expression of virulence genes and occurrence of antisense RNAs as observed in the Δrho mutant. Growth of S. aureus was not affected by BCM treatment. In the presence of BCM higher levels of SaeSR-dependent transcripts were identified by using northern blot analysis and microarray expression data. Similarly, the secretome analysis revealed higher amounts of SaeSR-dependent virulence factors. In further experiments we found that the Δrho mutant exhibited increased virulence in a murine infection model. Taken together, these findings suggest that BCM potentially has an impact on staphylococcal virulence.

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Virus-induced transition from pneumococcal biofilm colonization to infection provides leads to novel vaccine strategies

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Streptococcus pneumoniae and Staphylococcus aureus are two human opportunistic pathogens of the respiratory tract that effectively colonizes the nasopharynx. When exposed to changes in the nasopharyngeal environment, induced by virus infection and its resultant inflammation, colonizing bacteria of both species can disperse from their biofilm environment, disseminate, and transition to cause infection. The mechanisms of pneumococcal biofilm dispersal are currently not well-established but clues from the bacterial transcriptome and preliminary experiments in vitro and in vivo suggest a role for bacterial proteases and possibly other enzymes. Virus-related biofilm dispersal is associated with a marked change in the bacterial transcriptome geared towards protection of the bacteria against the host immune system with increased expression of established virulence determinants (capsule, PspA, Ply, etc), bacteriocins and carbohydrate metabolism. Therefore, bacteria exposed to virus-associated inflammation, are highly inflammatory and provides a potentially more physiological model to investigate disease and preventive measures. Using transcriptional information, we have identified several novel conserved antigens, only expressed on disease-causing, biofilm-dispersed organisms, that when used for immunization show broad protection against otitis media, pneumonia and invasive pneumococcal disease, without eliminating or affecting pneumococcal colonization. Thus, a better understanding of pneumococcal and Staphylococcal disease progression provides us with potential novel disease-directed preventive strategies that could eliminate the problems with serotype-replacement seen with current polysaccharide vaccines and are readily adaptable to other commensal pathogens in the future.

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Visualization of Haemophilus influenzae sialic acid theft from primary human bronchial epithelial cells and selective inhibition with sialic acid-based inhibitor abrogates serum resistance

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Non-typeable Haemophilus influenzae (NTHi) is a commensal organism part of the upper respiratory tract microbiome, but can become an opportunistic pathogen in children and elderly. For instance, NTHi infections are found in more than 50% of children suffering from otitis media, an inflammatory disease of the middle ear that affects 65-300 million people globally and is a major cause of hearing loss. In addition, NTHi is found in the majority of patients with acute exacerbation of chronic obstructive pulmonary disease, a leading cause of death worldwide. The switch of NTHi from a symbiotic colonizing bacterium to an opportunistic pathogen is associated with an increased resistance to serum-mediated killing. A key virulence factor in this process is the uptake and presentation of sialic acid sugars on the cell surface. The metabolic pathway for sialic acid utilization by NTHi is therefore a promising therapeutic target.
Here we report on a bacterial selective sialic acid-based inhibitor (SiaNAc-3Fax) that prevents sialic acid incorporation into NTHi lipooligosaccharide at high nanomolar concentrations and enhances serum-mediated killing of NTHi. In an in vitro model of the human respiratory tract, we demonstrate efficient inhibition of sialic acid transfer from primary human bronchial epithelial cells to NTHi using bioorthogonal chemistry. Since no vaccines exist for NTHi, the efficient and selective inhibition of NTHi LOS sialylation by SiaNAc-3Fax and its effect on serum resistance may represent an alternative therapeutic strategy to treat NTHi infection.

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Novel Double-Attenuated Influenza A Live Vaccines in Swine

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Pigs are commercially relevant livestock and frequently infected with influenza. This disease is of great economic relevance and bears high zoonotic risks. To reduce disease burden and occlude virus reservoirs, effective vaccination has remained a key issue. However, the conventional inactivated vaccines often provide insufficient levels of protection. By reverse genetics, we generated a double-attenuated mutant of the strain A/Bayern/74/2009 (H1N1v), carrying a non-physiological, strictly elastase-dependent HA cleavage site and a C-terminally truncated NS1 protein. In-vitro, the double-attenuated mutant strain showed a strictly elastase-dependent growth. Additionally, we investigated its potential to serve as a live vaccine in swine. Prime-boost immunized animals developed neither clinical symptoms nor nasal virus shedding if challenged with the homologous wild-type. Furthermore, we observed considerably reduced clinical signs and no shed virus in nasal swabs after homo-subtypic infection with another unrelated H1N1 strain. Overall, strong protection elicited by the elastase HA cleavage site/NS1 mutant against the same HA/NA subtype suggests broad tolerance against antigenic drift.

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Antimicrobial activity in sputum from Intensive Care Unit (ICU) patients

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Introduction: Streptococcus pneumoniae and Staphylococcus aureus are known causative agents of ventilator-associated pneumonia (VAP), which leads to increased morbidity and mortality of intensive care unit (ICU) patients. The objective of this study was to investigate the potential antimicrobial activity in sputum samples collected from different ICU patients. We hypothesized that antimicrobial activity could be protective against pneumonia, but that the patient's outcome may also be influenced by other patient parameters. The present studies were aimed at establishing an ex vivo assay that mimics the in vivo situation through incubation of bacteria with the sputum samples.

Material and methods: Sputum and clinical metadata were collected from 53 mechanically ventilated ICU patients from the University Medical Center Groningen. Antimicrobial activity in sputum was tested via a sputum spotting assay, where sputum was spotted on blood agar plates with confluent lawns of S. pneumoniae, S. aureus or a Streptococcus anginosus isolate from sputum. Upon overnight incubation, growth inhibition zones were compared. To assess the possible impact of the sputum's microbiome on growth inhibition, bacterial sputum isolates were analyzed in a similar spotting assay.

Results and Conclusions: The sputum spotting assay revealed major differences in the bactericidal activity of sputa from different patients. Intriguingly, the bacterial spotting assay did not show similar inhibitory effects on pneumococcal growth. This implies that, in particular, host factors and therapeutic interventions determine the antimicrobial activity in the sputum of mechanically ventilated patients.

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Intranasal vaccination with lipoproteins confers protection against pneumococcal colonization

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Surface exposed lipoproteins of pneumococci, covalently anchored to the cell membrane, are highly conserved among pneumococcal serotypes and were evaluated for their protective potential in vitro and in vivo. A multiplex-based immunoproteomics approach revealed the immunogenicity of selected lipoproteins when administered to mice indicating high antibody titres for MetQ, PnrA and DacB. The analysis of convalescent patient sera confirmed the immunogenicity especially for lipoproteins PnrA, PsaA, and DacB. Testing the surface abundance by flow cytometry using mouse antisera indicated that the most abundant proteins were PpmA, PnrA and DacB. In a mouse model of colonization mice were intranasally immunized with PnrA, DacB and MetQ followed by intranasal challenge with pneumococci. PnrA induced strong protection in a murine model of pneumococcal colonization. Immunization with DacB and MetQ also led to a reduction in bacterial load in the nasal cavity, although to a lower degree. Importantly, reduction in bacterial recovery correlated with increased production of antigen-specific IL-17A in the nasal cavity. In contrast, protection was only partially accompanied by high mucosal and systemic antigen-specific IgG titres. In summary, we could show that lipoproteins are interesting targets for future vaccine strategies as they are highly conserved, abundant and immunogenic. Using different screening methods we finally identified PnrA, DacB and MetQ as potential vaccine antigens to induce protection against pneumococcal colonization which in turn will lead to a decline in transmission.

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Therapeutic perspectives in pneumococcal pneumonia: From molecule to treatment

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Pneumonia is the most frequent infectious disease worldwide, being the leading infectious cause of death in children worldwide and causing a tremendous socioeconomic burden in industrialised countries. Importantly, despite appropriate antibiotic treatment, 14–35 % of all hospitalised community-acquired pneumonia (CAP) patients die, depending on age and co-morbidities. Thus, a great medical need for the development of adjuvant therapeutic strategies in addition to antibiotics is evident.
Dysregulation of the innate immune system drives lung injury and its systemic sequelae due to breakdown of vascular barrier function, harmful hyperinflammation and microcirculatory failure, which contribute to the unfavourable outcome of patients with severe pneumonia. A variety of promising therapeutic targets have been identified and numerous innovative therapeutic approaches demonstrated to improve lung injury in experimental preclinical studies. However, at present specific preventive or curative strategies for the treatment of lung failure in pneumonia in addition to antibiotics are still missing. The aim of this talk is to give a short overview of some novel adjuvant therapeutic strategies for pneumonia and its most important complications, sepsis and acute respiratory distress syndrome, to explain their molecular rationales and briefly discuss future perspectives.

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Pathogenesis and transmission of the novel highly pathogenic avian influenza H5N8 2016 virus in ferrets and mice.

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Background and objectives: The incursion of new reassortants of HPAI H5N8 resulted in epidemic outbreaks among wild birds and poultry throughout Europe. In this study, the potential of cross-species infection was evaluated in mammalian animal models.
Materials and methods: The virulence and pathotype of A/tufted duck/Germany/AR8444/2016 H5N8 was assessed by experimental inoculation of ferrets and Balb/c mice. Viral titers in nasal washes or organ samples were determined. Sero-responses were measured by ELISA (NP and H5 proteins) and HI assay.
Results: The H5N8 virus replicated in mouse lungs and spread systemically to the brain without prior adaptation. An infectious dose of 102 TCID50/animal resulted in a survival rate of 40 %, while animals receiving higher dosages had to be euthanized until 9 DPI. Therefore, HPAIV H5N8 replicate in mice and demonstrated a high-pathogenicity phenotype.
Ferrets were i.n. inoculated with 106 TCID50/animal with naive ferrets served as transmission sentinels by direct contact. No respiratory symptoms and only minor changes in body weight and body temperature were detected. From a single nasal wash sample virus was re-isolated, and moderate viral titers were determined from organ samples. The contact ferrets did not seroconvert.
Conclusion: Clade 2.3.4.4 H5N8 virus exhibited a mildly virulent phenotype in ferrets and was not transmissible, consistent with lack of reported human cases, while it demonstrated high-pathogenicity in mice.

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The ecology of MERS-CoV: From host reservoir to disease

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In 2012 a novel coronavirus associated with severe respiratory disease in humans emerged in the Middle East. To date, over 2000 cases of MERS-CoV have been reported, with an approximate case fatality rate of 35 %. Circulation of MERS-CoV-like viruses has been identified in various bat species. However, epidemiological investigations identified dromedary camels as the likely source of MERS-CoV zoonotic transmission. Neutralizing antibodies, viral RNA and infectious virus have been detected in dromedary camels throughout the Middle East and Africa. Zoonotic and human-to-human transmission occurs relatively frequently. Human-to-human transmission occurs predominantly in hospital settings. No prophylactic and therapeutic countermeasures against MERS-CoV in humans are currently available. Several vaccine development programs for MERS-CoV are currently underway, including vectored and classical subunit approaches. A potentially promising way to minimize the public health impact of MERS-CoV is to prevent transmission from dromedary camels to humans. Here we present data on the ecology of MERS-CoV in relationship to the ancestral and current host reservoir, transmission, disease in humans and the development of countermeasures.

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Virulence determinants of recent German avian influenza isolates subtype H7N7 in different host species

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Avian influenza viruses (AIVs) belong to the family Orthomyxoviridae and have a segmented, single stranded RNA genome coding for at least ten viral proteins. The two membrane proteins hemagglutinin (HA) and neuraminidase (NA) enable the differentiation in 16 HA and 9 NA distinct subtypes. Most of the viruses remain low pathogenic (LP) carrying a HA with a monobasic cleavage site (CS) which is activated by trypsin-like proteases and hence, cause mild infections. Some H5 and H7 evolve to high pathogenic (HP) phenotypes from LPAIV precursors by the requirement of a polybasic CS within the HA. This motif is recognized by ubiquitous furin-like proteases and leads to systemic infections with up to 100 percent mortality. To investigate necessities for the shift from LP to HPAIV a natural pair of H7N7 from an outbreak in Germany 2015 was analyzed. Using reverse genetics four viruses were generated: LP, LP with a polybasic CS (LP_poly), LP carrying HA from HP (LP_HPHA) and HP. Pathogenicity of these recombinants was examined by performing animal experiments with chickens, turkeys and ducks. While the ducks stayed healthy independent from infection route and virus, chickens and turkeys showed severe symptoms and 100% mortality for the HPAIV. Interestingly the chickens seemed to be more susceptible than the turkeys towards LP_poly and LP_HPHA but both viruses were less virulent than HP. Hence, we added single segments of the HP to LP_poly and LP_HPHA for investigation in animal trials. The NS segment seemed to be essential for high virulence in chickens.

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Mutations in conserved NA residues of H5N1 naturally isolated from humans modulated sialidase activity and virulence in mice but not in chickens

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H5N1 of clade 2.2.1 is endemic in Egypt since 2006 and 2 distinct clades have evolved: clade 2.2.1.1 in commercial poultry and clades 2.2.1.2 and 2.2.1.2a in humans and poultry. Compared to the neuraminidase (NA) of the parental 2.2.1 viruses, avian viruses in clade 2.2.1.1 possessed one mutation (I168T) and human-like viruses in clades 2.2.1.2 and 2.2.1.2a had 4 mutations (A46D, L204M, S319F and S430G) and 16 mutations, respectively. Here, recombinant 2.2.1.2a viruses carrying different NA resembling those in clade 2.2.1, 2.2.1.1 or 2.2.1.2 or single mutations were generated. In vitro, no or minimal impact on replication in cell cultures, plaque size, cleavability, receptor binding activity (RBA) and oseltamivir resistance was observed. Viruses with human-like NA had significantly lower NA activity than viruses with avian-like NA. Reduced NA activity of 2.2.1.2a was due to L204M. Insertion of L204M in three HxN1 viruses also reduced the NA activity. Over 97% (n=8053) of NA sequences in the GenBank possessed L204. All inoculated chickens died within 3 dpi. In mice, virus with L204M exhibited lower virulence and did not kill all animals, whereas S319F and S430G increased the virulence without remarkable difference in the cellular immune response. Together, H5N1 viruses in humans acquire NA mutations to maximize fitness in mammals without impact on replication in poultry.

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Pathophysiological role of the nucleoside substrate-binding protein PnrA of Streptococcus pneumoniae

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Introduction: Streptococcus pneumoniae produces a large repertoire of ABC transporters to maintain bacterial fitness and virulence in its various ecological niches in humans. The pnr (pneumococcal nucleoside receptor) operon is constituted of pnrA, the other genes of the Pnr ABC transporter and the upstream genes are annotated for nucleoside metabolism. PnrA is a pneumococcal lipoprotein and substrate-binding proteins (SBP) of the nucleoside PnrABC transporter system. Here we introduce the structure of PnrA and we further delineate its role in the pathophysiology of pneumococci.
Results: Growth experiments showed no difference for the pnrA-mutant in the complex medium THY and chemically-defined RPMI 1640modi. medium compared to the isogenic wild-type. Flow cytometric analysis and immunoblots after protein fractionation indicated the surface localization of PnrA and release into the supernatant upon inactivation of the diacylglyceryl-transferase Lgt. Structural analysis indicated similarities to the purine receptor TmpC of Treponema pallidum (1). Phagocytosis experiments revealed a higher number of recovered intracellular pneumococci of the pnrA-mutant when compared to the wild-type. IN accordance, immunofluorescence microscopy demonstrated a higher uptake of pnrA-mutant bacteria, while intracellular survival was identical to the isogenic parental strain. In the acute pneumonia model mice infected with the PnrA-deficient pneumococci showed, although not significantly, improved survival, while the pnrA-mutant virulence was not similar to the wild-type in the sepsis model.
Conclusion: Pneumococcal PnrA, highly abundant on the cell surface, is important for pneumococcal fitness in the respiratory tract, where limitations of nutrients require intact substrate uptake systems.

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Reversing erythromycin resistance in GAS isolates using HAMLET- a breast milk protein-lipid complex

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Drugs that once used to treat infections, are becoming less effective. Therefore, treatment alternatives are becoming limited and drug resistant infections are becoming one of the major causes of death worldwide. Among these infections is the flesh-eating disease caused by group A Streptococci (GAS). In this kind of infection, penicillin is the treatment of choice, but when penicillin allergy is a hinder, erythromycin is used instead. Although no penicillin resistant strain has been detected, erythromycin resistant isolates using different resistance mechanisms have emerged and the death incidence rates are increasing. In this project, we aim to reverse resistance to erythromycin and other antibiotics in GAS clinical isolates through a purified human breast milk protein-lipid complex termed HAMLET. In vitro, short-time kill assays showed reduced bacterial growth along with limited bacterial survival in isolates treated with HAMLET alone. Mechanistically, fluorescence measurement indicated membrane depolarization and bacterial cell death events taking place in isolates treated with high [HAMLET]. At lower concentrations, cell death is reduced but membrane depolarization still takes place. Based on these results, we speculated that the increase in membrane depolarization seen at low [HAMLET] could facilitate the internalization of antibiotics and increase their bactericidal activity. Using the MIC assay, combined HAMLET and erythromycin treatment of GAS isolates confirmed our hypothesis and reduced the erythromycin MIC rendering them sensitive again regardless of the inherent resistance mechanism. These preliminary results present HAMLET as a possible antibiotic adjuvant to treat and limit antibiotic resistance in GAS infections.

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PNA as antisense therapeutics in Streptococcus pyogenes

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Streptococcus pyogenes (Group A Streptococcus, GAS) is a Gram-positive and strictly human pathogen which causes mild infections of throat and skin as well as serious diseases including necrotizing fasciitis. GAS is exquisitely sensitive against penicillin, but many patients are allergic to this antibiotic. For this reason, novel therapeutic therapies must be developed like peptide-conjugated antisense-peptide nucleic acids (PNAs). PNAs are small molecules which contain a pseudo-peptide backbone and can be linked to cell penetrating peptides (CPPs) for improved bacterial uptake. In our study, we targeted gyrA with different CPP-conjugated antisense PNAs. To test the effect of the PNAs, killing assays and RT-qPCR were performed. Galleria mellonella was used as GAS infection and therapy model. Galleria mellonella larvae were infected with GAS and subsequently treated with anti-gyrA PNAs. In killing assays (KFF)3K-anti-gyrA-PNAs as well as TAT-anti-gyrA-PNAs showed a dose-dependent effect in GAS. In contrast, anti-gyrA-PNAs coupled to Antennapedia and Cadherin CPPs did not kill the bacteria. RT-qPCR revealed that the gyrA transcript level was reduced to about 50 % at sub-killing PNA concentrations. Larvae treated with TAT-anti-gyrA-PNAs showed an increased survival in comparison to larvae treated with scrambled anti-gyrA-PNAs. Survival of the larvae could be further increased by administration of a PNA/Levofloxacin combination. We could show that peptide-coupled antisense PNAs are effective in GAS and that the Galleria mellonella model can be applied as an in vivo prescreening model for therapeutic agents.

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Von Willebrand Factor represents a new adhesion cofactor of Streptococcus pneumoniae

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Streptococcus pneumoniae is the main cause of community acquired pneumonia. Former own studies reported the induction of Von Willebrand factor (vWF) secretion by pneumococcal adherence to endothelial cells. VWF is mediating platelet adhesion and thrombus stabilization at injured endothelium. As globular glycoprotein, vWF is circulating within the blood and also forms long high molecular weight fibers on activated endothelial surfaces exposed to strong shear stress.
The main topic of this project aims to analyse the interaction between S. pneumoniae and human vWF.
The present comprehensive analyses indicate binding of pneumococcus to both, globular vWF proteins and to multimerized vWF fibers, generated on the endothelial cell surface. Iodinated vWF protein demonstrated variations in binding activity to various pneumococcus serotypes. Furthermore, flow cytometry analyses revealed a strong dependence of vWF binding on amount of capsule polysaccharides. The binding of vWF to floating pneumococci as well as by cell surface-adhered pneumococci is repeatedly detected and quantified by immuno fluorescence microscopy with specific antibody detection. In order to mimic the mechanosensitive conformational change from a globular vWF into vWF-fibers, binding to pneumococci was analysed using primary human endothelial cells in an Ibidi® microfluidic system. Using this system, bacterial attachment to long vWF fibers for more than 20 min was observed.
The presented vWF-binding data demonstrate that pneumococci recruite vWF for attachment to blood vessel walls even in the bloodstream at high shear rates.

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Signals involved in the transition from colonization to disease with Streptococcus pneumoniae

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Streptococcus pneumoniae (the pneumococcus) effectively colonizes the human nasopharynx and does so by forming well-organized biofilm communities. Transition to disease occurs frequently enough that the pneumococcus remains a leading cause of otitis media, pneumonia, sepsis, and meningitis worldwide, resulting in millions of infections and deaths annually. Changes in the local environment caused by virus infection or host assaults triggers biofilm dispersal from colonizing biofilm pneumococci. The resulting dispersed pneumococcal population is distinct in its transcriptome and phenotype as compared to both biofilm bacteria and their broth-grown planktonic counterparts, displaying increased virulence and invasiveness as well as inducing more host inflammation. However, the exact mechanism(s) of transition to disease has been less studied. Here, we utilize previously established in vitro biofilm formation and biofilm dispersal models to investigate how pneumococcal bacterial egress from biofilms occurs after recognition of increased temperature (i.e., mimicking fever). Dampened biofilm dispersal in the presence of protease inhibitors implicate a potential role for proteases in heat-induced egress from biofilms. We identify a role for serine/protease HtrA in heat-induced biofilm dispersal, as biofilms formed by a HtrA-negative mutant were unable to respond to heat exposure as compared to wildtype, albeit the formed biofilms were comparable in biomass and structure. In vivo experiments utilizing our previously established mouse models are ongoing. Understanding the specific mechanisms involved in the transition to pneumococcal infection provides novel strategies to specifically interfere with disease progression without disturbing normal colonization of the nasopharynx.

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Proteome analysis of Streptococcus suis under stress conditions and in host-pathogen interaction

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Streptococcus suis is a Gram-positive zoonotic pathogen which causes septicemia, meningitis, arthritis and further diseases in its natural swine host as well as in humans. At present, there are 35 serotypes identified, based on their capsular polysaccharide (1). The mechanisms involved in the pathogenesis and virulence of S. suis are only partially resolved. A better understanding of virulence factors and pathophysiology of S. suis is needed to improve therapeutic strategies. We aim to investigate conserved and broadly distributed proteins to screen for new candidates for multicomponent vaccines. In this work, we compare the proteome composition of specific S. suis serotypes under different physiological conditions and from infection settings. Secreted proteins in the supernatant were enriched by protein precipitation, while cells were mechanically disrupted to obtain protein extracts. Protein analysis of different samples was carried out using a Q Exactive Plus Hybrid Quadropole-Orbitrap mass spectrometer. Using a DIA approach with a mass spectral library we will comprehensively cover the proteome of S. suis and gain excellent reproducibility in quantitation to identify proteins specifically expressed in vivo. So far we cover 1200 proteins of three different S. suis serotypes. These data are now being used to establish a first spectral library. Further studies like immunoproteomics experiments are necessary to evaluate the use of identified virulence factors as a vaccine candidate for swine.

(1) Fittipaldi N. et al. (2012), Future Microbiology, 7, 259-279.

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1-Methyltryptophan improves the outcome of murine polymicrobial sepsis

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Pneumonia is a common cause of systemic bacterial dissemination complicated by organ failure (sepsis) or even septic shock. Sepsis is still associated with astoundingly high morbidity and mortality despite improvements in intensive care. It is characterized by a complex systemic immune response with two phases: hyperinflammation and hypoinflammation. The regulation of the immune response involves the degradation of tryptophan (Trp) by indolamin-2,3-dioxygenase (IDO) via the kynurenin (Kyn) pathway. IDO is highly active during sepsis and the pathway has immunosuppressive effects. In our study, polymicrobial sepsis was induced by the acute peritonitis model in C57BL/6 mice. The treatment group received 1-methyltryptophane (1-MT) in the drinking water to inhibit the activity of IDO. IDO activity was assessed via the Kyn/Trp ratio in serum, which was measured using tandem mass spectrometry.
Prophylactic administration of 1-MT reduced the high mortality due to sepsis from 50 % to 20 %. In search of an explanation, the effects of 1-MT treatment alone were analyzed prior to or in the absence of sepsis induction. 1-MT pre-treatmet (i) lowered the expression of MHC-II on antigen-presenting cells, (ii) pre-activated CD4+ T cells as shown be induction of the cell surface markers CD69 and CD25, and (iii) activated regulatory T cells. How these 1-MT effects confer protection from subsequent sepsis remains to be determined.

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Characterization of the respiratory and gastrointestinal microbiota during a bacto-viral co-infection in swine and mouse – A multi-omics approach

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Having access to appropriate pathogenicity models is crucial to investigate host-pathogen interactions during bacto-viral-co-infections, which are characterized by their high mortality. Albeit mice are widely used as infection models, they are hampered by their limited physiological similarity to humans. Due to its large accordance to humans regarding its physiology and its immune system, swine has become an attractive alternative. Recent studies indicate a clear correlation between structure and function of the human microbiota, progression of infections and consequently also the host health status. Therefore, we investigate the gastrointestinal and respiratory tract microbiota of swine and mouse under healthy conditions and after a co-infection with influenza A and Streptococcus sp. Elucidation of structure and function of the microbiome during a bacto-viral-co-infection will allow identifying molecular markers on the genome, transcriptome and proteome level. As a starting point, we are establishing an integrated analyses pipeline to enable a direct comparison of meta genomics, transcriptomics, and proteomics data. To this end, stool as well as nasal- and bronchoalveolar lavage samples will be divided for parallel DNA, RNA and protein extraction followed by state-of-the-art 16S rRNA gene-sequencing, transcriptional analyses and mass-spectrometry. First results indicate that thorough homogenization of the fecal samples is one of the major challenges for establishing a sample preparation pipeline with high quality outcome. Until now, best results were achieved by mechanical homogenization of samples frozen in liquid nitrogen. We are currently optimizing and evaluating a TRIzol-based protocol by comparing this approach to other well-established protocols for meta-omics sample preparation.

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Metabolic and regulatory adaptation of Staphylococcus aureus to different carbon sources

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Different parts of the human body are characterized by specific carbon sources available to S. aureus for growth and proliferation. For example, during invasive disease glucose is the major carbon source in the blood stream, whereas pyruvate may correlate with a colonizing lifestyle since it is available in significant amounts in nasal secretion. Lactate for instance is produced in high amounts by proliferating T-cells at the site of infection and may therefore function as a signal of the hosts' state of immune defense. Finally, for many intracellular pathogens glycerol has been demonstrated as a major carbon source during host cell invasion and thus may have an equally important role for S. aureus during intracellular survival which is an important step in the development of chronic S. aureus infections.
For a better understanding of the S. aureus adaption processes during life as a harmless commensal or as a pathogen during invasive disease, we analysed carbon source utilization of S. aureus strain COL using a combined metabolomic and proteomic approach. S. aureus was grown in a synthetic medium with glucose, pyruvate, lactate or glycerol as sole carbon source. To also investigate the impact of carbon catabolite repression in this process, we included a catabolite control protein A mutant in our analyses and exposed S. aureus to combinations of glucose with either pyruvate, lactate or glycerol. Absolute quantitative data will be presented describing the intra- and extracellular protein repertoire and metabolic profile of S. aureus when grown with different carbon sources.

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Determination of potential virulence of an AIV from subtype H4N2 with a polybasic cleavage motif within the hemagglutinin using reverse genetics and animal experiments

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According to the definition of the OIE highly pathogenic avian influenza viruses (HPAIVs) are naturally restricted to H5 and H7 subtypes. These HPAIVs possess a polybasic cleavage site (CS) in the hemagglutinin (HA) and have an intravenous pathogenicity index (IVPI) ≥1.2. In 2012, an H4N2 virus (Quail/CA12) with a polybasic CS 322PEKRRTR/G329 was isolated from quails in California. Here, we investigated the virulence of this virus in chickens after insertion of point mutations in the CS and reassortment with HPAIVs H5N1 and H7N7. All gene segments of Quail/CA12 were cloned in pHWSccdB vector. Using QuikChange® mutagenesis, threonine at position 327 in the CS was changed either to arginine (R327) or lysine (K327). In addition to the wild-type virus, viruses carrying R327 or K327 in the CS with or without gene segments from HPAIVs H5N1 or H7N7 were constructed. Moreover a mutant of Quail/CA12 possessing the CS of a highly pathogenic H5N2-strain was generated. Chickens were inoculated via intravenous (IV) or oculonasal (ON) routes and observed for 10 days. The H4N2 virus exhibited high virulence with IVPI > 2.0 when acquired non-HA genes from HPAIV H5N1 and HA gene with R327 or K327 plus other gene segments from HPAIV H5N1. The IVPI values of the other viruses ranged from 0 to 0.6 indicating low virulence. Together, the H4N2 virus may shift to high virulence after reassortment with contemporary HPAIV H5N1. Single mutations in the CS were not sufficient to shift to high virulence in the wild type.

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S. epidermidis secreted serine protease Esp as a new putative IgG4 and IgE-reactive protein in asthma patients

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It is well documented that exposure to bacteria is generally associated with protection from allergy. Certain bacterial species, however, can elicit the generation of specific T-helper type 2 cells as well as IgE and become targets of allergic immune responses. However, the nature of the bacterial allergens has remained elusive.
The aim of this study is to identify proteins in S. epidermidis which can induce Th2 immune responses in allergic patients. Transferring our successful approach from S. aureus to these bacteria, we employed sera of asthmatic subjects to conduct a systematic search for IgG4-reactive extracellular proteins in S. epidermidis. Using one-dimensional immunoblotting we observed IgG4 binding to some proteins in the sera of healthy and asthmatic individuals. A peak of around 31 kDa was observed in most reacting subjects. This corresponds to the molecular mass of S. epidermidis Esp. We generated recombinant Esp and analysed binding of IgG1, IgG4 and IgE in sera of healthy and asthmatic individuals. This revealed that the antibody response to Esp was indeed skewed toward IgG4, and the serum levels of specific IgE were higher in asthmatic than in healthy individuals.
Our findings indicate that Esp may be a bacterial allergen. They underline the power of our approach for candidate allergen discovery. We propose that allergic responses to colonizing bacteria could explain why some asthma patients have intractable symptoms despite full medical treatment. In this case our results could pave the way to new diagnostic tools as well as treatment strategies for asthma.

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Panoramic view on Staphylococcus aureus biofilm physiology

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Staphylococcus aureus represents a dangerous opportunistic bacterial pathogen causing a wide range of diseases like skin abscesses, bacteraemia and sepsis. S. aureus also forms biofilms on host tissues and implants leading to chronic infections, e.g. found in osteomyelitis, endocarditis and cystic fibrosis patients. Despite the facts that up to 80 % of human bacterial infections are biofilm-associated and Staphyloococci are recognized as the most frequent causes of biofilm-associated infections, little is known about biofilm physiology of S. aureus. In order to shed light on molecular key factors during S. aureus biofilm formation, we compared protein and metabolic profiles of planktonic and biofilm-associated cells using state-of-the-art omics technologies.

As a starting point, we established a flow-through system suited for highly reproducible cultivation of S. aureus biofilms and multi-omics analysis. Application of this system allowed us to analyse the intra- and extracellular biofilm proteome and to determine concentration profiles of 39 utilized and secreted metabolites during biofilm growth. Based on 1736 quantified proteins, we found numerous proteins involved in anaerobiosis, nitrate respiration, arginine deiminase pathway, capsule biosynthesis, osmotic stress response and cardiolipin biosynthesis as well as toxins and proteases strongly induced during biofilm growth. Integration of metabolome data (e. g. accumulation of fermentation products like formate and lactate), biofilm formation assays, hemolysis assays and a Galleria mellonella infection model contributed confirming our proteome data.

In conclusion, integrated multi-omics data contribute to a better understanding of biofilm-associated S. aureus infections - an essential prerequisite for the development of novel antimicrobial therapies.

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The hidden lipoproteome of Staphylococcus aureus

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Lipoproteins are attached to the outer leaflet of the membrane by a di- or tri-acylglyceryl moiety and are thus positioned at the membrane-cell wall interface. Consequently, lipoproteins are involved in many surface associated functions, including cell wall synthesis, electron transport, uptake of nutrients, surface stress response, signal transduction, and represent a reservoir of bacterial virulence factors. Inspection of 123 annotated Staphylococcus aureus genome sequences in the public domain revealed that this organism devotes about 2-3 % of its coding capacity to lipoproteins, corresponding to about 70 lipoproteins per genome. 60 of these lipoproteins were identified in 95 % of the genomes analyzed and were thus deemed the core lipoproteome of S. aureus. While 30 % of the conserved staphylococcal lipoproteins are substrate-binding proteins of ABC transporters with roles in nutrient transport, much less is known about the function of the remaining lipoproteins. Here, we summarize current knowledge and integrate information from genetic context analysis, expression and regulatory data, domain architecture, sequence and structural information, and phylogenetic distribution to provide potential starting points for experimental evaluation of the biological function of the poorly or uncharacterized lipoproteome of S. aureus. First results from a screening of 19 mutants with deletions in genes encoding so far uncharacterized lipoproteins will be presented suggesting a role for two of these proteins in cell division.

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Phenotypical effects of lipoteichoic acid deficiency in Streptococcus pneumoniae

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The structure of pneumococcal wall teichoic acid (pnWTA) and lipoteichoic acid (pnLTA) differ from of other Gram-positive bacteria in several aspects. Pneumococcal teichoic acids (pnTAs) are composed of identical and complex repeating unit structures suggesting a common biosynthetic pathway. These sugar polymers are highly decorated with non-covalently bound phosphorylcholine (P-Cho), which is essential for growth. Based on in silico analysis of S. pneumoniae genomes, putative enzymes involved in biosynthesis of teichoic acids have been proposed. Allelic replacement of the S. pneumoniae D39 gene spd_1672 (tacL – teichoic acid ligase), encoding for an enzyme with homology to O-antigen ligases from Gram-negative bacteria, was conducted to test the hypothesis that TacL is essential for anchoring pnLTA to the pneumococcal surface. Chemical analysis of the pnTAs of TacL-deficient mutants indicated a total loss of pnLTA but not pnWTA. Phenotypic characterization of D39ΔtacL revealed only a minor impact on growth in chemically-defined medium and a slightly increased autolysis. The cell morphology of pneumococcal wild-type, mutants and complemented mutants was visualized using SEM and TEM, while the quantities of selected choline-binding proteins (CBPs) and the capsule content were investigated by flow cytometry. The effect of TacL deletion on adherence or phagocytoses was analyzed using human epithelial cells (A549) or phagocytes (PMA-differentiated THP-1 cells). Finally, mouse infection models were used to evaluate the role of LTA during pneumonia or sepsis. The loss of TacL resulted in an unaltered phenotype, but in a significant attenuation, indicating an essential role of pnLTA in the pathophysiology of pneumococci.

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Investigation of the phosphoproteome of Streptococcus pneumoniae

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Due to the fact that resistance to antibiotics is emerging rapidly in pneumococci it is of great relevance to analyse the pneumococcal proteome. Data about protein abundance in Streptococcus pneumoniae may provide an extensive source of information to facilitate the development of new vaccines and drug treatments. It is known, that protein phosphorylation on serine, threonine and tyrosine residues is a major regulatory post-translational modification in pathogenic bacteria. For this reason it is of particular interest to gather precise qualitative and quantitative information about the proteome and especially the phosphoproteome of the pneumococcus. Thus, the unencapsulated D39 strain, a kinase (ΔstkP) and phosphatase mutant (ΔphpP) were analysed in a label-free global proteome quantification experiment using GeLC-MS/MS. Moreover, the phosphoproteome was investigated using SILAC followed by enrichment of phosphorylated peptides with titan dioxide and mass spectrometric analysis. The morphological characterisation of all strains by electron microscopy revealed abnormal cell division and cell separation in both mutants. Label-free data point out several protein groups that are regulated contrary in ΔphpP and ΔstkP: for example proteins involved in cell division, peptidoglycan biosynthesis, DNA replication and pyrimidine metabolism are higher abundant in ΔphpP but lower abundant or not changed in ΔstkP. Proteins belonging to fatty acid biosynthesis are regulated oppositely. These results already support the assumption that the phosphatase PhpP and kinase StkP are forming a functional signalling couple. Commonly identified phosphorylated proteins include cell division proteins FtsZ and DivIVA. Furthermore, phosphorylation sites in some ABC-transporters and cation transporters were identified so far.

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Influence of the two-component regulatory system 09 on adaptive processes of Streptococcus pneumoniae

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Pathogenic bacteria have evolved sophisticated mechanisms to multiply and survive under various environmental conditions. The human pathobiont Streptococcus pneumoniae encounters highly successful different host compartments. Critical steps during pneumococcal colonization and dissemination within the human host are its adaptive mechanisms to changing conditions. Here two-component regulatory systems (TCS) are of major importance as they are sensed by environmental signals. The role of pneumococcal TCS has only partially elucidated, however meanwhile the scant information on TCS09 suggests an association with nutrients sensing. Here we aimed to elucidate the role of TCS09 under planktonic and infection-related conditions. Isogenic mutants for Δrr09, Δhk09 and both TCS09 components (Δtcs09) were generated in D39 and characterized in functional assays in in vitro and in vivo experiments. We identified that the TCS09 is involved in an effective defense and sensing mechanism against oxidative stress by regulating the CcdA-Etrx1-MsrAB2 (CEM) system (Saleh et al., 2013; Ribes et al., 2016). Pneumococcal mutants deficient in HK09 or the complete TCS09 were reduced in their capability to survive extracellular oxidative stress. Furthermore, the regulation of pneumococcal adhesins CbpL and PspC via the TCS09 was investigated by flow-cytometry. Results revealed a lower surface expression in all generated mutants. Nonetheless, no decreased adhesion to eukaryotic lung epithelial cells was observed. A moderately increased survival was observed for mice infected with tcs-mutants using an in vivo mouse pneumonia infection model. In conclusion, these data strongly suggest that the TCS09 responds to oxidative stress conditions pneumococci are faced during dissemination.

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Laboratory Mice Are Frequently Colonized with Staphylococcus aureus and Mount a Systemic Immune Response - Note of Caution for Murine Infection Studies

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So far, laboratory mice were not considered as natural hosts of Staphylococcus aureus, and their suitability for S. aureus infection and vaccination studies has been questioned. We previously isolated a mouse-adapted S. aureus strain, causing infections in laboratory mice. Therefore, we wondered whether laboratory mice are commonly colonized with S. aureus and whether this might impact on infection experiments. Health reports from commercial vendors revealed that colonization with S. aureus is common, with rates as high as 21% among specific-pathogen-free mice. We genotyped 230 S. aureus isolates from various vendor facilities (Charles River facilities in the USA, Canada, France, and Germany; another US vendor), and university- or company-associated breeding facilities in Germany, China and New Zealand. Finally, we quantified the antibody response in naïve and S. aureus-exposed mice. Three S. aureus lineages had spread globally among laboratory mice and accounted for three quarters of the isolates: CC1 (13.5 %), CC15 (14.3 %), and CC88 (47.0 %). Compared to lineage-matched human isolates, the murine isolates frequently lacked hlb-converting phages, superantigen genes on mobile genetic elements, and ampicillin resistance, implying long-term adaptation to the murine host. Importantly, natural S. aureus colonization induced a systemic IgG response against many S. aureus proteins, including several vaccine candidates. In conclusion, laboratory mice are natural hosts of S. aureus and, consequently, could provide better infection models than previously assumed. Pre-exposure to the bacteria represents a possible confounder in S. aureus infection and vaccination studies. Hence, we encourage researchers to use S. aureus-free or consistently primed mice

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Proteomic response of Streptococcus pneumoniae to treatment with 2,2'-bipyridine – an iron limitation approach

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The human pathogen Streptococcus pneumoniae is able to cause several disease ranging from mild types like otitis media to life-threating forms such as pneumonia or bacteraemia, especially in young, elderly and immunosuppressed patients. Although there are vaccines against certain pneumococcal serotypes available, not all are covered which leads to serotype replacement. This strengthens the need for protein based vaccines. Hence, it is important to understand the pneumococcal virulence on protein level. Application of in vitro infection models helps to gain insight into infection processes in the human host. Iron is an essential trace element, which is involved in various key metabolic pathways but is extremely limited within the human host. Bacteria evolved many different mechanisms to acquire iron from its environment. In this study, the pneumococcal proteome response to the iron chelator 2,2'-bipyridine (BIP) was investigated. Moreover, two different media were analyzed to examine the influence of the initial iron concentration on protein regulation after iron limitation. Interestingly, proteins involved in pathogenesis showed a different regulation in media compared. In iron-poor medium proteins of this group were down regulated whereas these proteins are up regulated in iron-rich medium. However, iron limitation in both environments led to a strong up-regulation of the iron uptake protein PiuA and the significant down-regulation of the non-heme iron-containing ferritin Dpr. On the base of these results it is hypothesized that the pneumococcal proteome response to BIP treatment is strongly dependent to the initial iron concentration in the medium or the environment.

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Interplay between glutamine and arginine on nitrogen metabolism in Streptococcus pneumoniae

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Streptococcus pneumoniae is a common colonizer of the human nasopharynx and can cause severe upper respiratory tract (URT) and systemic infections. During colonization and infection, pneumococci need to adapt to different host environments with regards to the availability of nutrients. Previous studies revealed that pneumococci are auxotrophic for several amino acids such as glutamine, glycine, cysteine, or histidine and that these amino acids are supplied by specific ABC transporter systems. Strain D39 has six glutamine transporters and deletion of the GlnHPQ (spd1098/1099) resulted in loss of fitness and reduced virulence (Härtel et al., 2012). To study the importance of glutamine as nitrogen source, we performed isotopologue profiling with 15N-labeled glutamine. After growth in chemically defined medium (CDM), 40-50 % of 15N-labeled glutamate, alanine and proline, and 20-40 % of aspartate, threonine, leucine and isoleucine was detected demonstrating that these amino acids are synthesized out of glutamine. Arginine is metabolized in strain TIGR4 by the arginine deiminase system generating ornithine, NH4+, CO2 and ATP. This is regulated by the activator ArgR2 controlling the arcA-C and arcDT genes (Schulz et al., 2014). Recently, ArcD was analysed as ornithine/arginine antiporter. Data base comparison revealed that ArcT has similarity to peptidases or deacetylases. Deletion of arcT in TIGR4 showed a reduced growth in CDM. To study ArcT in more detail, ArcT was heterologous expressed and purified. An enzyme assay was carried out with rArcT and Nα-acetyl-L-ornithine as substrate. By HPLC analysis, we could determine ornithine indicating that rArcT can act as N-acetylornithine deacetylase.

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Regulation and production of human contact factors during streptococcal infection

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The human contact system – also known as the intrinsic pathway of coagulation or kallikrein/kinin system – consists of four plasma proteins: factor XII (FXII), plasma kallikrein (PK), factor XI (FXI) and high-molecular-weight kininogen (HK). The contact system is activated as a response to bacterial infections, which supports it`s contribution to the early innate immune defense against bacteria. The mammalian liver responds to infection by a dramatic change in the synthesis of various plasma proteins. Here we study the regulation of contact factors during an infection with Streptococcus pyogenes, an important human pathogen, in vitro by using the human hepatoma cell lines HepG2 and HROHep03, and in vivo in S. pyogenes infected mice by analysis of the mRNA levels via real-time PCR. Our data show an increased production of contact factors after 24 h of infection.
Bacteria like Streptococcus pyogenes are also associated with sepsis, which is a serious medical condition caused by an overwhelming immune response to the infection. It is characterized by systemic signs and symptoms of inflammation. We analyzed the contact factor levels of twenty sepsis patients by ELISA and substrate assay to determine the contact factor concentration and to analyze the FXII and PK activity in plasma of survivor and non-survivor patients during the first three days of disease.

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Impact of the polysaccharide capsule and pneumolysin on pneumococcal biofilm formation

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Biofilms allow bacteria to colonize various biotic and abiotic surfaces, protect against environmental conditions like the immune system and create niches for horizontal gene transfer. A wide range of medical important bacterial species exhibit the ability to form biofilms during colonization and infection of the host. Streptococcus pneumoniae, one of the main causative agents of severe human respiratory tract infections, was shown to form biofilms in vivo (Chao et al. 2014). To study the impact of pneumolysin (ply) and the polysaccharide capsule (cps), two of the main pneumococcal virulence factors, different GFP-expressing wild-type strains and their isogenic cps and ply mutants were analyzed for the ability to form biofilms. Therefore, three different systems with chamber slides for the analysis of static and semi-dynamic biofilm conditions and a continus flow-system for the evaluation of pneumococcal biofilms grown under defined dynamic conditions were established. The formation of pneucmococal biofilms was analysed byFluorescence, confocal and electron microscopic analysis of. Thus, we were able to visualize on the one hand differences between the analyzed pneumococcal wild-type strains (D39, R36A, C080) and on the other hand we show that the pneumococcal capsule as well as the presence of pneumolysin have a direct influence on the amount and the structure of the formed biofilm.
In future studies, we will use the established systems for the analysis of pneumococcal gene regulation during the process of biofilm formation. Furthermore, small-scale proteomics of re-isolated pneumococci will help to characterize the biofilm grown bacteria in comparison to planktonic grown pneumococci.

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Insights into the metabolomic inventory of Streptococcus pneumoniae

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The pathogen Streptococcus pneumoniae can cause a broad range of severe diseases like pneumonia, septicaemia etc. Deciphering its metabolome would improve our understanding of its patho-physiology and its dependency on host-derived nutrients. We aimed for a metabolomics approach that would allow us to investigate basic metabolic characteristics during growth in a CDM. To analyze the complex variety of metabolites we used analytical techniques like 1H-NMR, HPLC-MS, GC-MS. Extracellular metabolites like carbohydrates and amino acids were quantified. We monitored important nutrients like glucose, adenine and asparagine but we detected also accumulation of fermentation products, and other metabolites. With the first sampling protocol for intracellular metabolome analysis for pneumococci in hand we can show for the first time a basal snapshot of the intracellular pneumococcal metabolome. By using HPLC-MS and GC-MS, nearly 100 intracellular metabolites, including amino acids, carbohydrates, nucleotides and organic acids, were identified. The intracellular metabolic profile shows high abundances for ATP and Frc-1.6-bP, most likely due to the high glycolytic metabolic flux. Other high abundant metabolites are UTP and UTP-dependent precursors of peptidoglycan synthesis probably to fuel cell-wall-metabolism. Our data clearly demonstrate the importance of distinct biochemical pathways for pneumococci such as glycolysis and lactate fermentation as well as its dependency on external host derived nutrients such as purines and pyrimidines. These essential pathways may display new targets in the drug development against pneumococci. By comparing metabolic stress profiles of drugs with known and unknown targets, a pathway analysis might reveal new modes of action for antimicrobials.

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Serotype distribution and antibiotic susceptibility patterns of Streptococcus pneumoniae among children less than five years of age in Accra, Ghana

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Background: Globally, Streptococcus pneumoniae causes about 11 % of all deaths in children under five years of age. The majority of these children are from developing countries like Ghana, where the epidemiology of pneumococcal carriage is poorly understood. This study investigated the serotype distribution and susceptibility patterns of S. pneumoniae among children younger than 5 years at a paediatric healthcare centre in Ghana.
Method: Epidemiological data on demographics, risk factors for pneumococcal carriage, and nasopharyngeal swabs were collected from 423 randomly selected children. The specimens were cultured and pneumococcal isolates were tested for antibiotic susceptibility and serotyped by latex agglutination. Multivariate analysis was conducted to determine association between risk factors and pneumococcal carriage.
Results: Pneumococcal carriage prevalence was 48.9 % (207/423) with highest carriage prevalence occurring in 7-12 months age group. In the multivariate analysis, pneumococcal carriage was significantly associated with runny nose (odds ratio = 1.9, p = 0.003) and day-care attendance (odds ratio = 1.5, p = 0.04). All the isolates were susceptible to ceftriaxone. However, resistance to cotrimoxazole, ampicillin, tetracycline, penicillin and erythromycin were 100 %, 88 %, 78 %, 63 % and 24 % respectively. Fourteen different pneumococcal serogroups/serotypes were identified and serogroup 6 was the most prevalent (30 %), followed by serotype 19 (20 %).
Conclusion: Pneumococcal carriage among the children was high, with increased resistance to several antibiotics. Ceftriaxone is a suitable antibiotic for treating pneumococcal infections in Ghana. The introduction of a pneumococcal vaccine is expected to significantly reduce the burden of pneumococcal disease in Ghana.

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Wild rodents and shrews are natural hosts of Staphylococcus aureus

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Laboratory mice are the most often used animal model for Staphylococcus aureus infections and are frequently colonized with this bacterium as we have previously shown. Since laboratory mice originate from wild house mice, we investigated whether wild rodents, including house mice, as well as shrews are naturally colonized with S. aureus and whether the bacteria adapt to the wild animal host.

295 animals of ten different species were caught in different remote locations over four years (2012–2015) in Germany, France and the Czech Republic.

45 animals were positive for S. aureus (15.3 %); three were co-colonized with two different isolates, resulting in 48 S. aureus isolates in total. The S. aureus isolates grouped into six lineages (Clonal complex (CC)49, CC88, CC130, CC1956, sequence type (ST)890, ST3033). CC49 isolates were most abundant (17/48, 35.4 %), followed by CC1956 (14/48, 29.2 %) and ST890 (9/48, 18.8 %).

The wild animal isolates lacked some properties commonly found among human isolates, e.g., a phage-encoded immune evasion cluster, superantigen genes on mobile genetic elements and antibiotic resistance genes. This suggests long-term adaptation to the wild animal host.

Nevertheless, one CC130 isolate contained the mecC gene, implying wild rodents might be both reservoir and vector for methicillin-resistant S. aureus.

In conclusion, we demonstrated that wild rodents and shrews are naturally colonized with S. aureus, and that those S. aureus isolates are likely host-adapted. Whether those strains might be more suitable for mouse infection experiments than the commonly used human-adapted strains is under investigation.

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An immunoproteomic approach to identify protein candidates for pathogen detection and risk stratification in sepsis patients

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Sepsis, "as life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer M et al. 2016), is the third leading cause of death in hospitalized patients in Germany. One reason for the high mortality is an often missing or delayed identification of the causative agent.

We aim to complement conventional microbiological and PCR-based diagnosis by developing an immunoassay for the quantification of the antibody binding kinetics to several bacterial proteins simultaneously.

In a prospective clinical trial (IMI_Sep, Greifswald) we collected plasma samples of patients before the onset of sepsis, at diagnosis and during the infection. For screening, patient plasma antibody binding to the extracellular proteins of the corresponding sepsis pathogen was quantified using a Simple Western Assay (ProteinSimple®). For patients with the highest increase of plasma antibody binding during sepsis, two 2D immunoblots were performed to compare antibodies in plasma taken before sepsis onset with those in plasma obtained at a later time point. Immunogenic proteins were identified by mass spectrometry.

During the disease course, often new spots appeared on the 2D immunoblots and intensities of other spots increased. For each bacterial species five particularly immunogenic proteins were selected and will now be cloned, recombinantly expressed and included in a multiplex assay (suspension array).

Such a multiplex immunoassay may complement the microbiological and sequence-based diagnosis of sepsis-causing agents as well as be suitable for risk stratification of sepsis patients. It also promises new insights into the role of the adaptive immune response in infection control.

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Characterization of clinical isolates using the pan peptidome and SWATH-MS

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The usage of data independent approaches (DIA) in modern proteomics permits comprehensive peptide identification/quantification results avoiding missing values in large sample cohorts commonly inherent for classical shotgun proteomics. Despite this paradigm shift in proteomics, analysis of DIA datasets is highly dependent on suitable spectral libraries working as a register that can be used for data mining of the DIA spectral inventories. Usage of DIA methods in high throughput studies of clinical isolates was precluded so far as these methods are restricted to previously measured data mostly performed for model organisms.
To overcome this limitation, we seek to adapt the already widely accepted pan genome principle for registers of spectral libraries in order to develop a pan peptidome approach allowing for thorough analyses of clinical strains.
In frame of our project a pan spectral library of four S. aureus laboratory strains is built. As central part of our proposed workflow using the DIA implementation OpenSWATH it results in a faster and more precise method for peptide identification and quantification combined in a new approach for characterization of clinical isolates and common strains.
For a 'proof of principle' we processed experimental samples from strains included in pan proteome and clinical S. aureus isolates (source: University Hospital, Greifswald).

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Detection of low frequency B cells specific for immunodominant virulence factors of Staphylococcus aureus

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The use of monoclonal antibodies for passive vaccination could be promising in the treatment of severe Staphylococcus aureus (S. aureus) infections. Whereas several monoclonal antibodies were found to mediate protection in mice, similar trials in humans failed. Therefore, new strategies to develop human-derived monoclonal antibodies against certain staphylococcal antigens are necessary. A crucial step for generating human monoclonal antibodies against single S. aureus antigens ex vivo is the isolation of specific B cells.
Therefore we selected four conserved and immunodominant S. aureus proteins: glycerophosphodiester phosphodiesterase (GlpQ), alpha-toxin (Hla), phosphatidylinositol-specific phospholipase C (Plc) and serine protease-like protein B (SplB). These proteins were recombinantly produced in Escherichia coli BL21, to be used as S. aureus antigens in further assays. Specific IgG levels were determined in the sera of 10 healthy donors by ELISA.
PBMCs were isolated from donors with high antibody concentrations. Since circulating B cells do not spontaneously secrete antibodies, the isolated B cells were pre-stimulated with R848 and IL-2 for 3 days. The frequency of antigen-specific B cells was determined by B cell-ELISpot assays. One out of 10,000 to 500,000 B cells was specific for the respective tested antigen. Because of this low frequency we intend to enrich those antigen-specific B cells in vitro and detect them by flow cytometry. Once isolated, these cells will be used to produce human monoclonal antibodies by means of single cell sequencing of the immunoglobulin variable regions as well as genetically engineering a vector encoding the antigen-specific antibody for expression in HEK293 cells.

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The gate and its keepers: Responses of epithelial cells and invariant T cells in bactoviral co-infections in pigs

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The epithelium between nasal cavity and lung represents an enormous environmental contact area and hence is the primary target for invasion of respiratory pathogens like Influenza viruses or Streptococcus suis. Cellular responses at this first line of defense are pivotal for the initial recognition of infection as well as the induction of a specific immune response. We established five immortalized cell lines from porcine epithelial tissues along the infection route from nose to lung. In these, we investigate the immediate cellular response upon infection. However, pathogen clearance is accomplished only by specialized immune cells.

The relatively abundant invariant T cells close the gap between early innate and late adaptive responses, thereby disrupting the pathogen's potentially deadly advantage. In contrast to conventional T cells, they express a semi-invariant T cell receptor recognizing bacterial structures and metabolites presented on MHC-like molecules. Responses from invariant T cells are readily inducible and include production of pro-inflammatory cytokines or cytolytic clearance of infected cells. Of the two invariant T cell subpopulations – invariant Natural Killer T cells (iNKT) and Mucosal-associated invariant T cells (MAIT) – only iNKTs have been described in swine. Using a combination of flow cytometry and PCR-based methods, we aim to describe MAITs and investigate the responses of both populations in monocausal as well as in co-infections. These investigations will further deepen the understanding of infectious respiratory diseases in swine. Since swine physiologically and immunologically closely resemble humans, the study additionally supports the establishment of this new biomedical model.

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Bacterial and viral coinfections: a multidisciplinary and multispecies approach to unravel signatures of respiratory infections

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Bacterial and viral co-infections of the respiratory tract are life-threatening and present a global burden to the global community. Staphylococcus aureus and Streptococcus pneumoniae are frequent colonizers of the upper respiratory tract. Imbalances through acquisition of seasonal viruses, e.g. Influenza A virus (IAV), can lead to bacterial dissemination to the lower respiratory tract, which in turn can result in severe pneumonia. The mechanisms behind the synergies between IAV and bacteria are poorly understood.
Here, we aim to decipher the progression of the disease, host-pathogen interactions at the site of infection, immune responses, tissue pathology, and microbiome plasticity in response to bacterial and viral mono- and coinfections. Therefor, three different in vitro and in vivo approaches will be employed. First, in vitro infections of human immune and lung cells in monolayer cultures and a more advanced tissue engineering approach of human lung will be analyzed. Second, global analyses, including proteomics and transcriptomics, of immune responses locally and in circulation, immune cell composition in blood and mucosal tissues, lung pathology, and disease progression from colonization by bacteria to severe pneumonia as a result of subsequent IAV infection will be employed in mice. Third, identical global transcriptomic and proteomic analyses will be performed by utilizing swine as a new biomedical in vivo model of infectious diseases. Therefor, techniques such as advanced microscopy, FACS, FLEXMAP and RNAseq as well as SRM mass spectrometry will be used. This multidisciplinary approach will unravel signatures of respiratory infections, including onset of disease and severe outcome.

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Volatile emission of bacteria frequently involved in coinfections

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Introduction: Bacterial and viral coinfections represent a crucial problem in clinical medicine. Volatile emissions may help to recognize organism growth and coinfections non-invasively. In order to understand these complex systems VOC profiles from typical coinfection agents such as Streptococcus pyogenes have to be investigated with respect to proliferation phases.
Method: VOCs from headspaces above bacterial cultures were preconcentrated by means of needle trap devices (NTDs). Compounds were thermally desorbed from NTDs and analysed by GC-MS. VOC analysis was done on different time points after inoculation. Bacterial growth was assessed through determination of optical density of bacterial cultures. At every time point, headspace of three bacterial cultures was investigated. In parallel, three media samples were taken as control.
Results: VOC profiles mirrored bacterial growth kinetics. Some VOCs were only detectable in the headspace of S. pyogenes and not in the medium. Several substances accumulated after 5 and 6 hours. After this maximum, concentrations of most substances decreased again. First accumulation of VOCs took place when growth of Streptococcus pyogenes changed from exponential to stationary growth phase.
Discussion: Time course of VOC patterns mirrored active adjustments of metabolic pathways during bacterial proliferation. VOC analysis could be used for non-invasive monitoring of bacterial growth and could, therefore, indicate infection. VOC profiles from pure cultures could be compared with profiles from coinfections including S. pyogenes. Differences in emitted substances and their concentration could thus show changes and interactions during the process of coinfection.

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Comparison of Staphylococcus aureus-induced pneumonia in BALB/c vs. C57BL/6 mice

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Staphylococcus (S.) aureus is one of the most important causes of nosocomial pneumonia. The high incidence and severity of respiratory infections with methicillin-resistant S. aureus strengthens the need for new preventive and therapeutic approaches.
Mice are the most commonly used animal model for S. aureus infection and vaccination studies. It is known that mouse strains differ in their immune response and hence susceptibility to S. aureus infection. Here, we compared the immune response of the two most commonly used inbred mouse strains, BALB/c and C57BL/6, to S. aureus-induced pneumonia.
We infected BALB/c and C57BL/6 mice intranasally with S. aureus Newman and analyzed the survival, the bacterial load in the lungs, as well as the local cytokine and chemokine levels 48 hours post infection.
Infected BALB/c and C57BL/6 mice did not differ in the survival rate and the bacterial load in the lungs. Pro-inflammatory cytokine levels (IL-2, TNF, IL-6) were comparable between both mouse strains, whereas IFN-γ and IL-22 were slightly higher in C57BL/6 mice. Surprisingly, typical Th2 cytokines (IL-4, IL-5, IL-13, IL-21) were not detected - neither in C57BL/6 nor in BALB/c mice. Pneumonia also triggered a strong chemokine production in the lungs with higher levels of the chemokines BLC (type 2 response) and, unexpectedly, MIP-1α (type 1 response) in BALB/c.
In conclusion, there were differences in the immune response of S. aureus-induced pneumonia due to the genetic background of the mice. However, in our model these differences did not translate into different susceptibilities to S. aureus pneumonia.

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Lesion profile in raptors monitored during the 2016-2017 H5N8 outbreak in Northern Germany

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During winter 2016-2017, Germany was affected by an outbreak of highly pathogenic avian influenza (HPAI; H5N8 influenza A virus), which caused mass mortality in poultry and various species of wild birds. This study was performed in order to unravel the potential thread of H5N8-induced HPAI on raptors.
Therefore 34 raptors (15 white-tailed sea eagles, 14 common buzzards, 2 northern goshawks, 1 rough-legged buzzard, 1 red kite, and 1 peregrine falcon) collected in Northern Germany between 9th November 2016 and 30th March 2017 underwent necropsy under biosafety level 3 conditions followed by histopathological, immunohistological and virological investigations. Morphologic lesions consistent with HPAI, influenza A virus nucleoprotein-antigen and/or H5- and N8-specific RNAs were detected in the majority of the raptors, and led to a diagnosis of HPAI as the major reason of disease and/or death. Among the raptors with confirmed HPAIV infection, characteristic gross lesions were scarce and included hemorrhages, typhlitis, and myocarditis. In contrast, histopathological findings were common and included encephalitis, myocarditis, splenitis and typhlitis. Real time RT-PCR revealed H5- and N8-specific RNAs in brain and/or lungs.
Gross lesions are scarce in H5N8-induced HPAI in raptors and correct diagnosis relies on histopathological, immunohistological and virological confirmation. The spectrum of wild bird species affected by the 2016-2017 H5N8-virus differs from a previous HPAIV H5N1 epizootic in 2005-2006. In particular, multiple white-tailed sea eagles succumbed to H5N8-induced HPAI. HPAIV H5N8 infection therefore represents an emerging threat for this endangered species.

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Role of glycolysis during Streptococcus pneumoniae death induced by the human milk protein-lipid complex (HAMLET)

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HAMLET, a complex of alpha-lactalbumin (ALA) and oleic acid from human milk, kills Streptococcus pneumoniae by a mechanism that bears resemblance to apoptosis in eukaryote cells. To identify HAMLET's possible targets in pneumococci, we employed a proteomic approach that identified several potential candidates including two glycolytic enzymes, fructose bis-phosphate aldolase (FBPA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Treatment of pneumococci with HAMLET resulted in an immediate inhibition of both ATP and lactate production, suggesting an inhibitory effect on glycolysis. This was further supported by experiments showing that HAMLET's activity was partially inhibited by activation of glycolysis in the presence of high concentrations of glucose and that inhibition of glycolysis by 2-deoxyglucose made pneumococci more sensitive to HAMLET. This was not seen in pneumococci lacking a functional glycolytic pathway through genetic inactivation of GAPDH, suggesting GAPDH to be an important target for HAMLET activity. Both HAMLET and ALA (but not oleic acid) bound directly to both enzymes in solid phase assays and effectively inhibited their enzymatic activity. However, alpha-lactalbumin alone showed no toxic activity and did not block glycolysis in whole cells, suggesting a role for the oleic acid in the HAMLET complex in entering the bacterial cells for HAMLET to reach its target(s). The results of this study suggest that part of HAMLET's antibacterial activity relates to its ability to target and inhibit glycolytic enzymes, and is a first example of glycolysis targeting by an antimicrobial agent.

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Investigation of virulence determinants in highly pathogenic H10 avian influenza viruses using reverse genetics and animal experiments

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Avian Influenza Viruses (AIV) cause significant economic losses in poultry and can infect humans causing mild respiratory to severe or fatal symptoms. AIV's belong to the family of Orthomyxoviridae with 8 single-stranded negative-sense RNA-segments. According to the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) AIV's are classified into 16 HA- and 9 NA-subtypes. In chickens, all AIV's are low pathogenic (LP); however some H5 and H7 viruses can be highly pathogenic (HP). HP H5 and H7 viruses (1) possess a multibasic cleavage site (CS) in the HA, (2) have an intravenous pathogenicity index (IVPI) ranged from 1.2 - 3.0 and/or (3) grow in cell culture without exogenous trypsin. Exceptionally few H10 viruses have an IVPI >1.2 and are therefore classified as HPAIV. Moreover, H10 viruses infect a wide range of host species from birds to mammals. In this project, the virulence determinants of HPAI H10N4 (isolated form turkeys in England in 1979) and H10N5 (isolated from mandarin ducks out of a European consignment in Singapore in 1993) will be determined in chickens. Both showed organ tropism and induced death of a part of the tested birds in first historical experiments. Using reverse genetics, we will swap gene segments from HPAIV and LPAIV H10 and inoculate chickens to pin point the gene segment or mutation(s) responsible for the high virulence of these viruses. Furthermore, pathogenicity of European H10 viruses in mice and ferrets will be studied. Results will be helpful to understand the evolution of (non-H5/H7) HPAIV.

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Identification of IgG4-binding extracellular proteins of *Pseudomonas aeruginosa* in cystic fibrosis patients

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P. aeruginosa is a prominent cause of infection in cystic fibrosis (CF) patients of whom approximately 80 % are infected by this microorganism. It has been proposed that impairment of Treg function combined with a shift from a Th1/Th17- to a Th2 profile contributes to lung inflammation in CF. The underlying mechanisms, however, and especially the bacterial antigens responsible for skewing the immune response in CF remain elusive.The main goal of this project is identify IgG4-binding proteins by using *P. aeruginosa* bacterial isolates and sera obtained from cystic fibrosis patients. IgG4 serves as a proxy for IgE.
Initially, a systematic search for serum IgG4 binding to extracellular proteins of *P. aeruginosa* was conducted by one-dimensional immunoblotting. Next, serum IgG1- and IgG4 binding to the extracellular bacterial proteome are being performed by 2D immunoblotting using selected patient sera that contain specific IgG4. IgG4 binding protein spots are being digested and analysed by mass spectrometry.
Preliminary results show that the following protein candidates were identified as possible IgG4 targets in *P. aeruginosa:* alkaline metalloproteinase, chitin-binding protein, elastase, major porin, structural outer membrane porin and the protease LasA.
Although further analysis are needed, these results show that *P. aeruginosa could* release several IgG4 reactive proteins. The next step will be to determine whether these proteins are really IgG4-binding proteins and can elicit a Th2/IgE response in CF patients. Evidence for an allergic component in the anti-bacterial immune response would add a new dimension to diagnosis and treatment of CF.

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Imaging-based analysis of pathomechanisms in bacto-viral coinfection

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Viral infections of the respiratory tract often pave the way for bacterial secondary infections, which can heavily influence disease outcome in both humans and swine. As such, coinfections of influenza A viruses (IAV) and pneumonia-causing bacteria like Streptococcus pneumoniae and Staphylococcus aureus pose a large threat not only to human but to global health. Therefore, it is critical to understand pathogen-host- and pathogen-pathogen-interactions to elucidate disease progression and immune response in bacto-viral coinfection. In order to achieve spatio-temporal resolution of bacto-viral coinfection processes in disease-relevant porcine tissues and in vitro cell cultures, infection models are currently being established that allow high-resolution imaging of the pathogens and their cellular environment. This includes systematic evaluation of suitable GFP expression strategies for recombinant IAVs for live-imaging in polarized cell cultures and in vivo tracking of fluorescent protein-expressing viruses. Obstacles in high-resolution confocal live-imaging of infected, polarized cell cultures in transwell inserts and tissue imaging are addressed with customized in vitro cultivation devices and adaptation of state-of-the-art fluorescence-compatible tissue clearing techniques. At the end, the developed techniques and strategies will be used to track bacto-viral coinfection processes and host responses in infected swine tissue and organotypic in vitro air-liquid interface (ALI) cell culture models.

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The struggling way to characterize porcine innate lymphoid cells

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Innate lymphoid cells (ILCs) are a recently identified immune cell population of great interest nowadays. This heterogeneous group of innate immune cells seem to have beneficial and harmful functions in regulating immune responses, regaining tissue homeostasis and diseases. As innate immune cells, they occur primarily on interfaces of the respiratory and gastro-intestinal tract. First murine studies with viral and bacterial lung infections indicate that ILCs are critical for the repair of the epithelium as well as combating pathogens.
Within the framework of the KoInfekt Consortium, we want to establish the swine as a biomedical model for bacto-viral infections. ILCs as part of the early defense against infections influence numerous aspects in the porcine immune response to Influenza A virus (IAV) and bacteria. To date humans/mice are the only described species in which ILCs could be detected. We assume that the swine, whose immune system resembles that of humans for more than 80 %, also possess ILCs. At first, we will characterize porcine ILCs by establishing flow cytometry staining panels for distinctive cell surface markers. At present, a huge gap exists between tools available for humans/mice and the field of veterinary research. The extent of porcine antibodies is extremely limited currently. Our approach to detect porcine ILCs will be a combination of antibody-based negative cell selection and PCR verification of distinctive cell markers. The prospective main goal of the present study will be the clarification of ILC functions in mono- and co-infections with zoonotic pathogens (IAV; Streptococcus suis) in swine.

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