Open Projects 2012

Please note: this is only a preliminary list:

1.) Name: Dr. Amar Deep Sharma / Dr. Tobias Cantz
Institution: REBIRTH-Group Stem Cell Biology
Dept. Leader: Prof. Hans R. Schöler (MPI Münster)
Telephone: +49 (0) 511-532-5255
Email sharma.amar@mh-hannover

Name of two Co-supervisors (from different departments):
Prof. Dr. Michael Ott
Prof. Dr. Thomas Thum

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: Name of student(s): Bhavna Rani

Animal experiments involved: yes

Research focus:
Title: Analyses of microRNAs functions in liver
Aims: To investigate the role of microRNAs in normal and adult stem cell mediated liver regeneration
Funding: DFG

A healthy liver has a remarkable capacity to regenerate and to restore the liver mass completely after removal of up to 70% liver mass (1, 2). The molecular mechanism of liver regeneration involves complex network of genes. The expression of those genes is regulated at transcriptional and posttranscriptional levels. MicroRNAs are ~22 nucleotide long RNAs which regulate gene expression at posttranscriptional level. Till date, more than 900 human and 600 mouse microRNAs have been discovered. The broad range of effects of microRNA-mediated regulation has started to emerge in recent years from development, maintenance and carcinogenesis in liver.

Our previous work has revealed that microRNAs regulate apoptosis and regeneration in liver (3-5). Using mouse as a model system we identified significantly de-regulated microRNAs in early phase of liver regeneration. In the proposed project, we aim to identify microRNAs, which regulate the termination of regeneration in normal and adult stem cell mediated liver regeneration. Furthermore, we will investigate in vitro and in vivo function of identified microRNAs by gain and loss of function studies. Adeno-associated viral vectors would be used to modulate the expression of microRNAs in vivo. In addition, we would further explore the therapeutic potential of these microRNAs in the treatment of specific liver diseases.

Methods: MicroRNA cloning, quantitative real time PCR, Northern blots, cell culture, AAV production, partial hepatectomy in mice and immunohistochemistry.

Time schedule
1. During first year: MicroRNA profiling to identify deregulated microRNAs during late phase of liver regeneration. In addition, in vitro functional analyses by modulation of miRNAs in primary hepatocytes and hepatoma cells.
2. During second and third year: Overexpression and inhibition of microRNAs in mouse liver followed by liver regeneration analyses.
Group Members:

**Tobias Cantz**  Group leader

**Amar Deep Sharma**  Postdoc
Malte Sgodda  Postdoc
Irina Eberle  Postdoc
Bhavna Rani  PhD student
Reto Eggenschwiler  PhD student
Selina Möbus  PhD student
Abbas Beh-Pajooh  PhD student
Francoise André  Technician
Susanne Alfken  Technician

**Key References for project**

**Own references (selected):**
2.) Name: Dr. Christina Bade-Döding  
Institution: Dept. of Transfusion Medicine  
Dept. Leader: Prof. Dr. Rainer Blasczyk  
Telephone: ++(49) 511-532-9744  
Email bade-doeding.christina@mh-hannover.de  
Name of two Co-supervisors (from different departments):  
Prof. M. Messerle (Institute for Virology), Andreas Krüger (Immunology)  

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: x Name of student(s): Soumya Badrinath  
Lecturer: □; PhD Kommission: □; No, not yet: x  

Animal experiments involved: □ yes x no  

Research focus:  
Our work focuses on the selection and presentation of antigenic peptides through HLA class I molecules to the immune system. HLA class I molecules require the assistance of peptide loading complex (PLC) for efficient assembly in the endoplasmic reticulum (ER) [1]. The transmembrane glycoprotein tapasin (TPN) plays an important role in loading HLA class I molecules with high affinity peptides and thus has an indirect role in the immune recognition of certain pathogens [2]. Since TPN is a dedicated part of the PLC, it is an ideal target for viral interference [3].  
Few HLA class I molecules are able to load peptides independent of TPN, based on their polymorphism [4, 5]. During an infection, a viral immune evasion protein can differentially impact these HLA molecules and thus evade antigen presentation [6]. The HCMV US3 protein for example which is expressed during the early phase of HCMV infection retains TPN-dependent HLA molecules within the ER [7] and thus prevents the presentation of immunogenic epitopes to the immune system [8], whilst TPN-independent molecules remain unaffected [9].  
Viral immune escape mechanisms are complex and comprise the cellular and humoral immune responses. Interference of the virus with the function of antigen presentation results in poor or inappropriate activation of T cells. The immune evasion strategy of HCMV ensures that viral antigens are not presented on the cell surface during the early phase of HCMV infection [10], a critical time of replication and viral proliferation. The intracellular peptides presented through HLA molecules are targets for the immune response, thus the retaining of HLA molecules by HCMV proteins is for a virus the most effective strategy of immune escape.  
Since the expression kinetic of the HCMV HLA class I immune-evasion-genes is unknown, it is not yet well understood i) at what state of infection viral peptides are presented through TPN-independent HLA molecules, ii) the viral origin of those peptides and iii) the strength of the CTL response against a given viral-peptide/HLA molecule complex.  

Specific project and methods applied:  
Title: Impact of HCMV immune evasions on the repertoire of presented antigenic peptides  
Aims:  
In the proposed project we aim to investigate the expression kinetic of HCMV HLA class I immune-evasion-genes. Furthermore the peptide profile of TPN-independent HLA class I molecules with respect to different states of HCMV infection will be investigated.
Additionally we aim to answer the question if ligands of viral origin would be preferentially selected and presented, hence raising the question of immune competence of T cells. Structurally we aim to understand the basis of HLA-restriction and will solve the structures of viral-peptide-HLA complexes.

1. Expression kinetics of HCMV HLA class I immune-evasion-genes
This part of the project includes viral infection of target cells using HCMV strains following real time PCR to investigate levels of mRNA encoding viral proteins and detection of protein levels by Western blot.
   - S2 cell-culture
   - viral infection
   - RT PCR
   - SDS PAGE, Western blot

2. Profiling of viral peptides
Virus derived peptides that are presented through TPN independent HLA class I molecules will be detected by soluble HLA technology [11]. Target cells expressing truncated HLA molecules will be infected with HCMV virus. Peptides of affinity purified HLA molecules will be sequenced by mass spectrometry.
   - cloning of lentiviral vectors encoding for truncated sHLA molecules
   - transduction of target cells
   - S2 cell culture
   - sHLA quantification by ELISA
   - viral infection of sHLA expressing cells
   - large scale production of sHLA molecules in bioreactors
   - affinity purification of sHLA molecules
   - mass spectrometric sequencing of sHLA bound peptides

3. Measuring the competence of T cell immunity
Antigen presenting cells will be used as targets for cellular immune recognition. T2 cells lacking TAP proteins and thus not able to load peptides on their surface will be transduced with full-length constructs of TPN independent HLA class I molecules. Viral peptide, detected in part 2 of the project, will be synthesized and loaded onto the T2 cells. Cytotoxicity assays will allow detecting T cell frequencies and competence.
   - cloning of lentiviral vectors encoding for full-length HLA molecules
   - transduction of target cells
   - S2 cell culture
   - Peptide loading assays
   - Flow cytometric analysis
   - Magnetic isolation of T cell populations
   - Cytotoxicity assays

4. Crystallographic analysis of viral-peptide-HLA complexes
To understand the structural basis of viral-peptide-HLA complexes and their immunological influence on T cell recognition we will solve the structure of TPN independent HLA alleles bound to naturally presented viral epitopes.
   - Cloning of prokaryotic vectors encoding for truncated HLA molecules (vectors encoding for beta2-microglobulin are available)
   - Inclusion body preparations of HLA heavy chain and beta2-microglobulin

Zurück an: kruse.susanne@mh-hannover.de
- Refolding with HLA heavy chain, beta2-microglobulin and synthesized viral derived peptide
- Crystal tray setup, seeding from available crystals
- Solving the structures

**Time schedule**

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**Group Members:**
Soumya Badrinath, PhD student
Thomas Kraemer, Master student
Heike Schumacher, technician

Zurück an: kruse.susanne@mh-hannover.de
Key References for project


Own references (mainly 2009-2011):


Research focus:
The focus of our research is the regulation of immune responses in states of autoimmune inflammation, primary and secondary infections or a combination of both. Throughout the last years, the role of epithelial cells, both of the gut and the airways, in these processes has become increasingly recognized and has been a central interest of our studies. However, many questions regarding the detailed contribution of epithelia to immunity as well as disease development remain open. In the proposed project, we aim at clarifying how the physiology and immunological function of alveolar type II epithelial cells (AECII) is changed and possibly compromised by respiratory influenza A virus (IAV) infection.

Already in the context of the 1918/1919 influenza pandemic has it been recognized that influenza infections strongly predispose for severe secondary infections caused by a wide range of bacterial pathogens. Despite the availability of antibiotic treatments, this problem persists still today, evidenced by epidemiological studies during the 2009 swine flu outbreak. A number of reports published throughout the last years point at mechanisms by which the influenza virus modulates the host’s immune system so that effective anti-bacterial defense mechanisms are strongly compromised. Also roles for the airway epithelium in the synergism between influenza A virus and other pathogens are being increasingly discussed. For example, a loss of lung repair responses has been associated with enhanced susceptibility to the 2009 pandemic influenza A virus. There are additional concepts, in which receptors on airway epithelial cells, which are used by the secondary pathogens for adhesion, are up-regulated or differentially exposed in the virus infected lung.

Next to the synergism between infections with IAV and secondary bacterial pathogens such as Streptococcus pneumoniae or Staphylococcus aureus, also co-infection with several respiratory viruses are subject of epidemiological interest.

As the immunological function of the alveolar epithelium is mostly conferred by AECII, the proposed project focusses on how IAV infection alters the reactivity of these cells towards a subsequent encounter with additional bacterial or viral pathogens. We are currently working on the question of how AECII in the lungs of IAV infected mice influence the recruitment and function of effector cells such as NK cells, alveolar macrophages and neutrophils in response to a secondary infection with Streptococcus pneumoniae. To this end, we have successfully established a mouse co-infection model of these two pathogens. Additionally, other models of infections with pathogenic microorganisms such as Bordetella bronchiseptica, Staphylococcus aureus, Pseudomonas aeruginosa and Listeria monocytogenes are employed within the group, addressing different questions of immune regulation during infection and also autoimmunity. Regarding studies addressing the immunological function of AECII, we have extensive experience in the isolation of these cells from mice suffering from respiratory infections as well as from autoimmunity in the lung. Also subsequent functional analyses of AECII, such as gene expression analyses, have been performed to a large extent and have already revealed promising candidate genes for influencing response towards secondary infections. In a project completed within the previous term of the MD/PhD program (Andrea Autengruber), which addressed the phenotype of AECII during autoimmune disease in the lung, proteome analyses (in cooperation with Prof. L. Jänsch, HZI Braunschweig) revealed valuable insights and helped identify promising candidate factors for the identification of contributing pathways.
Specific project and methods applied:

Title: Insight the molecular network underlying influenza A-mediated modulation of alveolar type II epithelial cell responsiveness to secondary respiratory pathogens

Aims: In the proposed project the influence of influenza A virus infections on the responsiveness of AECII to secondary triggers supplied by pathogenic microorganisms will be addressed. We aim at gaining insight into the role of the airway epithelium in the synergism between IAV and secondary infections. The overall goal of the proposed project will be the identification of pathways critical in the modulation of airway responsiveness to secondary pathogens following IAV infection. Knowledge of such pathways will eventually allow the identification of new ways for therapeutic intervention of this clinically highly relevant phenomenon.

Funding: HGF

Based on our experience in AECII isolation and characterization, within the proposed project the following studies are to be performed in order to address the two following central questions:

Question 1: How does IAV infection alter the ability of AECII to respond to subsequent encounter of other viruses or bacteria?

Question 2: How does IAV infection alter AECII surface molecule expression to facilitate adhesion, colonization and infection by a secondary bacterial pathogen?

A The responsiveness of AECII of healthy mice towards various pattern recognition receptor (PRR) triggers derived from bacteria (e.g. LPS, CpG DNA) or viruses (e.g. poly I:C, R-848) will be addressed. For optimal control of experimental conditions, AECII will first be treated with these PRR ligands ex vivo before analysis. On the other hand, to better reflect the situation of a respiratory infection, mice will be treated with the ligands intratracheally, followed by AECII isolation and analysis. The AECII response will assessed by means of analysis of gene expression (microarray), secretion of soluble mediators (Luminex, ELISA) and the proteome. These investigations will yield a general overview on how AECII respond to different pathogen-related structures.

B To define the role of AECII in the reaction to respiratory infection with different classes of pathogens, the cells will be isolated and analyzed at different time-points after encounter. The reaction to the following pathogens will be addressed: Streptococcus pneumoniae, Bordetella bronchiseptica, Staphylococcus aureus, vesicular stomatitis virus and respiratory syncytial virus. These pathogens have been chosen to represent the two gram-positive bacteria that are most frequently found in patients suffering from bacterial superinfection following IAV (S. pneumoniae, S. aureus), gram-negative bacteria that cause not only acute but also chronic infections of the respiratory tract (B. bronchiseptica) and respiratory viruses. By performing the planned analyses in parallel for several pathogens, common pathways can be dissected from pathogen- or class-specific ones. Again, ex vivo and in vivo approaches will be pursued. First, isolated AECII will be co-cultured or infected with the pathogen and then analyzed for their responses. Depending on the class and strain of the respective pathogen we will be able to discriminate between reactions to intracellular and extracellular pathogens. In addition, analyses of AECII from animals intratracheally infected with the chosen pathogens will be performed. As in A, analyses will address the gene expression profile, proteome and secreted soluble mediators and will yield detailed knowledge on how AECII react to the encounter of different classes of respiratory pathogens.

C Based on the results of points A and B, in this part the question how IAV infection modulates AECII responsiveness to subsequent encounter with a secondary pathogen will be addressed. To this end, AECII isolated from IAV infected mice at different time-points post infection will be treated ex vivo with various PRR ligands and pathogens. The responses conferred by the AECII will be compared to those found in the previous parts of the project. Thereby it will be possible to assess how responsiveness is altered by a previous IAV infection. These analyses additionally aim at elucidating how this modulation depends on the timing of the underlying IAV infection and how the shape of the
AECII responses to secondary encounters of structures derived from pathogenic microorganisms changes during the course of the viral infection. In addition, to model superinfection in an IAV pre-infected host, AECII from animals subsequently infected with IAV and a secondary bacterial or viral pathogen at different time-points will be isolated and analyzed as described above. Importantly, as it has been suggested that the AECII phenotype is altered for a relatively long time after a viral infection and also that susceptibility to secondary bacterial infection lasts beyond the time-point of viral clearance, also analyses several weeks following IAV infection will be included. In combination with survival studies, these analyses will give insight on if and how altered AECII reactivity to secondary pathogens correlates with the outcome of co-infection.

Identified candidate pathways, which were found to contribute to the synergism between IAV and secondary infections, will be further characterized for their detailed role. Most importantly, it will be assessed whether blocking of pathways differentially activated or activation of possibly suppressed pathways will lead to a restoration of the physiological AECII responsiveness to the respective PRR ligand or even pathogen. In vivo studies using an adequate co-infection model will ultimately show whether interference with the identified candidates will be able to ameliorate enhanced susceptibility to a second pathogenic threat in the IAV infected host.

To address the second central question of the proposed project, i.e. whether surface expression of receptors critical for binding and infection of secondary bacterial pathogens on AECII is altered following IAV infection, AECII from IAV infected animals will be analyzed at several time-points post infection. These investigations will be comprised of microarray analyses of gene expression (focusing on surface proteins) and flow-cytometric analysis of surface molecules involved in the adherence of bacteria to the epithelium. In addition, AECII will be isolated from healthy mice or at different time-points post IAV infection and will be incubated with bacteria for subsequent microscopic analysis. These analyses will be performed in order to assess the capacity of the bacteria to bind to the cells and whether adherence is possibly better supported by AECII isolated from IAV infected mice. Candidates for surface molecules involved in enhancing susceptibility to bacterial infection following IAV infection will be further characterized in more detail. For example, blocking experiments will be performed where possible to assess whether bacterial adherence can be inhibited. Another strategy will be to use alveolar epithelial cell lines to knock-down candidate factors via siRNA transfection and assess the impact on bacterial binding.
Time schedule

1. Analysis of the responsiveness of AECII from healthy mice to various PRR ligands and respiratory pathogens. (1st year)

2. Analysis of the responsiveness of AECII to PRR ligands and pathogens at different time-points following IAV infection. Identification of pathways that are differentially activated depending on the viral pre-infection. Analysis of whether interference with the identified pathways is able to reverse the IAV-mediated detrimental modulation on the AECII responsiveness ex vivo and more importantly in vivo infection models. (2nd and 3rd year)

3. Analysis of the surface molecules differentially expressed by AECII at different time-points following IAV infection and their role in bacterial binding and impact on infection in vivo. (1st and 2nd year)

Group Members:
Dr. Sabine Stegemann-Koniszewski, Dr. Marcus Gereke, Dr. Andreas Jeron, Dr. Priya Sakthivel, Andrea Autengruber, PhD student; Harro Frauendorf, PhD student, Kathleen Rogge, PhD student, Julia Boehme, PhD student, Robert Weber, Bachelor student, Silvia Prettin, technician, Tatjana Hirsch, technician,

Key References for project

1. Bacterial co-infections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1) - United States, May-August 2009. MMWR Morb Mortal Wkly Rep 58: 1071-1074. mm5838a4 [pii].
10. Stegemann S et al. (2009) Increased susceptibility for superinfection with Streptococcus pneumoniae during influenza virus infection is not caused by TLR7-mediated lymphopenia. PLoS One 4: e4840. 10.1371/journal.pone.0004840 [doi].

Own references (mainly 2009-2011):


Zurück an: kruse.susanne@mh-hannover.de
Our research focuses on inflammation and autoimmunity in skin diseases, particularly in atopic dermatitis (AD). AD is a chronic and relapsing inflammatory skin disease that commonly begins in early infancy and is considered as the most common, itchy and relapsing inflammatory skin condition (for a review see Bieber 2010). The inflammatory skin infiltrate in AD lesions consists mostly of T helper cells, shaping the clinical symptoms, but recently also cytotoxic T cells are believed to be crucial for the development of eczema (Hennino et al. 2011).

First of all, exposure of allergic individuals to exogenous allergens leads to immediate type inflammation, but during the last years, our work contributed to the now well-accepted model, in which a T-cell dominated inflammation is associated with a cytokine switch from Th2 in the acute phase toward Th1 in the chronic phase.

Furthermore, allergic inflammation can persist in the absence of exposure to exogenous allergens, perpetuating the Th1-mediated inflammatory reaction. Several studies have gathered evidence supporting the idea that autoimmune mechanisms might contribute to the chronic manifestation (Valenta et al. 2009). During the last years, several human autoallergens have been identified, isolated or produced recombinantly. Autoallergens bearing sequence homologies to known environmental allergens (B-Ag) are discriminated from those, where no similarity can be observed (A-Ag). Concerning the class of B-Ag, our group could recently show a distinct cross-reactivity between a microbial antigen from Malassezia sympodiales and a human autoallergen, underlining the theory of sensitization due to a ‘molecular mimicry’ (Balaji et al. 2011).

While autoimmunity in AD has been investigated mostly on the level of humoral immunity (specific IgE), our group focuses on the role of T cells specific for autoallergens. During the last years, we could show in cooperation with Professor R. Valenta, Vienna, for the first time a T cell mediated immune response in patients with AD. This work on the A-Ag -NAC (Homs 2) was continued and using T cell clones from lesional skin and peripheral blood of patients with AD we could perform a characterization of autoallergen-specific T cells (Heratizadeh et al. 2011). Interestingly, 60% of T cell clones generated from peripheral blood turned out to be CD8+, while from lesional skin mostly CD4+ T cell clones could be generated.

Specific project and methods applied:
Title: Autoreactive CD8+ T lymphocytes in chronic inflammatory skin diseases
**Aims:** In the proposed project the role of autoreactive CD8$^+$ T cells in AD shall be investigated and necessary tools developed. Furthermore, different forms of AD shall be compared with regard to their set of autoreactive CD8$^+$ T cells.

**Funding:** LOM or HBRS (grant) and DFG (KFO250) for costs of experiments

T cell immunodominant peptide sequences of model allergens which function also as autoantigens shall be identified in a first step, aiming to the generation of MHC tetramers. The PhD thesis will start at a point when first MHC tetramers to autoallergens of interest will be available and specific T cells can be characterized in more detail: Recently, using overlapping nonamer peptides, we mapped two immunodominant regions within the autoallergen -NAC for presentation via MHC class I (HLA-A*0201) for presentation to CD8$^+$ T cells. Using these sequences, in cooperation with the NIH tetramer core facility and also via commercial acquisition, MHC tetramer and MHC pentamer complexes will be produced to be used for distinct staining of -NAC specific CD8$^+$ T cells. After identification of specific T cells using these unique reagents, a deep characterization will follow. To gain deep insight into the cells phenotype, in cooperation with Dr. Christian Hennig and Professor Gesine Hansen the Chipcytometry technique shall be applied, allowing staining of single cells with a set of multiple markers.

In order to map immunodominant regions of more antigens/allergens (Hom S 1-5) which are presented to CD8$^+$ T cells via MHC class I, nonamer peptides will be used to stimulate isolated T cells of sensitized patients. These nonamer peptides will be chosen either by software prediction tools or, in case of smaller proteins, covering the entire protein with an overlap of 3 amino acids. Since these smaller fragments may be bound by MHC class I and also class II, it will be necessary to detect specifically the proliferation of CD8$^+$ T cells by flow cytometry using CFSE as the proliferation marker. To underline the data derived from T cell proliferation testing, stabilization of MHC class II complexes on the cell surface of the human cell line T2 by peptide-binding is a suitable tool.

To investigate specific T cells regarding their cytokine profile, CD8$^+$ T cell lines and specific T cell clones of patients with AD and reactivity to an autoallergen will be established. Therefore, PBMCs of sensitized patients are stimulated with the antigen and grown under optimal culture conditions until limited dilution and final re-stimulation testing using the antigen and autologous APCs. Subsequently, the cytokine profile will be determined. The question shall be addressed, whether CD8$^+$ T cells have the potential to contribute to the inflammation and may have more than a bystander function in AD.

**Time schedule**
1. Generation of CD8$^+$ T cell lines and T cell clones from lesional skin and peripheral blood of patients with known sensitization to autoallergens in AD.
2. Epitope mapping of immunodominant regions of allergens and autoallergens.
3. Characterization of specific T cells, T cell lines and T cell clones to novel autoallergens using techniques described above.

**Group Members:**
Thomas Werfel, group leader, Lennart Rösner, PostDoc, Annice Heratizadeh, MD, Susanne Hradetzky, PhD student; Jenny Seltmann, PhD student; Gabriele Begemann, technician; Petra Kienlin, technician; Ute Staar, technician.
Key References (reviews) for project


Project related own references:


For more own references, please check Werfel-T in PUBMED.
5.) Name: Anna-Maria Dittrich, MD
Institution: Dept. of Pediatric Pneumology, Allergology and Neonatology
Dept. Leader: Prof. Dr. G. Hansen
Telephone: +49 (511) 532-9785
Email Dittrich.anna-maria@mh-hannover.de
Name of two Co-supervisors (from different departments):

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: x Name of student(s): Subhashree Mahapatra
Lecturer: X; PhD Kommission: ☐; No, not yet: X

Animal experiments involved: X yes ☐ no

Research focus:
Our laboratory is interested in understanding the immunological mechanisms that govern allergic sensitization in different allergic diseases with a focus on the development of new, individualized treatment strategies. We have recently shown that a pre-existing antigen-driven airway inflammation facilitates priming towards new antigens, regardless of the immunological phenotype of the inflammation. In that line, we identified IL-17 as a key mediator of airway sensitization facilitated by a Th1 or a Th17-polarized airway inflammation. In a transgenic murine model of Th17-dependent facilitation of secondary sensitization, we were able to show that a Th17-polarized airway inflammation not only facilitates priming towards new antigens but results in a phenotype of severe lymphocytic inflammation and airway hyperreactivity, features that bear a strong resemblance to the immunological phenotype observed in patients with severe asthma.

IL-17-dependent airway inflammation and the IL-17 dependent recruitment of neutrophils have been shown to be associated with severe asthma where disease outcomes are particularly poor and therapeutic options are limited. Therapeutic options in asthma achieve adequate asthma control in about 90% of the patients. However, this leaves approximately 10% of the patients who are not adequately controlled with the available treatment options. Additionally, none of the existing treatment modalities has been demonstrated to have distinct efficacy in the treatment of a Th17-dominated phenotype of bronchial asthma.

Given the association between increased IL-17 levels and IL-17 dependent neutrophil recruitment and the severity of airway inflammation, we believe that our model of Th17-dependent airway inflammation is uniquely suited to identify pharmacological treatment strategies with preferential efficacy in Th17-dependent airway inflammation, a clinical phenotype with currently unmet needs for better treatment options.

As other airway diseases such as cystic fibrosis, lung fibrosis, lung transplant rejection and others have also been shown to be associated with elevated IL-17 levels, we believe that the identification of pharmacological agents preferentially targeting IL-17 dependent airway inflammation will be an important step towards an improved, individualized treatment not only for patients with asthma but for many other lung diseases as well.

Specific project and methods applied:
Title: Pharmacological Intervention for Th17-mediated Airway inflammation
Aims: In the proposed project, we will identify pharmacological substances with superior efficacy in the treatment of Th17-dependent airway inflammation, compared to Th1- or Th2-dependent airway inflammation.
Funding: DFG

Zurück an: kruse.susanne@mh-hannover.de
We have recently developed models for transgenic OVA-induced Th1-, Th2- and Th17-dependent airway inflammation (Dittrich et. al. J Immunol. 2008). Furthermore, we have developed extensions of these models, which allow us to analyze the effects of pharmacological interventions on priming towards new antigens (Albrecht et. al. J Allergy Clin Immunol. 2011, and Figure 1). In these extended adoptive transfer models priming of endogenous T cells occurs with polarization corresponding to the initially transferred T helper cells. In conjunction, these models allow a direct comparison of the efficacy of different pharmacological agents in the treatment of differently polarized airway inflammation. Based on studies published by other groups, we will test three targets, a retinoic acid receptor agonist (AM80), an mTOR inhibitor (rapamycin) and an inhibitor of pyrimidine-synthesis (vidofludimus) which due to their known properties are likely to preferentially address Th17-dependent airway inflammation.

![Figure 1: Protocol for assessment of different agents on suppressing Th17-dependent airway disease during sensitization and effector phase.](image)

All agents will be tested comparatively in our models for Th1- Th2- and Th17-dependent airway inflammation in order to identify those with preferential activity against a Th17-dependent airway inflammation. Additionally, comparisons to standard anti-asthmatic treatment with dexamethasone will be performed.

In order to identify the mechanisms targeted by the different agents, we will administer the agents at different time points during our mouse models of Th-dependent airway inflammation and will analyze different read-out time points as graphically depicted in Figure 1.

A. To assess the efficacy of the agent for suppressing airway inflammation mediated by already polarized Th17 cells, we will treat mice at the time of transfer of Th1/Th2/Th17 polarized T cells and during the primary intranasal challenge with cognate antigen which induces distinct polarized airway inflammation. Airway inflammation will be analyzed by bronchoalveolar lavage and histology two days after the primary challenge with cognate antigen. Additionally, invasive lung function measurements will be performed to identify agents with efficacy against airway hyperreactivity. Cell cultures, flow cytometric and RT-CPR analyses as well as western blots of local and draining lymph node cells will analyze changes in cytokine profiles and T helper-associated transcription factor phosphorylation induced by the agents.
B. A second set of experiments will be performed to determine the efficacy of the agents in suppressing the endogenous priming and polarization of T helper cells. To this end, the agents will be administered as in A. However, analyses will take place only after the initial challenge with cognate antigen and a secondary, unrelated antigen and re-challenge with the secondary antigen alone after two weeks. Analyses will be as described in A, with the addition of measurements of antigen-specific serum immunoglobulins.

C. Finally, agents will be administered before the secondary challenge in order to assess the efficacy during the effector phase. Analyses will be performed as described in A and B.

**Time schedule**
1. Proficiency in T cell culture, animal treatment and read-out methodology will require app. 3 months.
2. Aim “A” will be started immediately after methodology is proficiently handled and will take approximately 9 months for different groups and agents.
3. Aim “B” and “C” can be started concomitantly with groups designed to serve for read-outs for “B” and “C” within the same experimental set-up. These will take app. 24 months.
4. Preparation of manuscript including aims “A” and “B” can be started concomitantly with ongoing experiments after 18 months and is expected to be finished after 30 to 36 months. Preparation of a second publication with results from aim “C” are expected to start at 30 months and to be finished by 36 months.

**Group Members:**
Subhashree Mahapatra, PhD student  
Maik Holme, MD-student  
Dr. Melanie Albrecht, Post-doc  
Dr. Sandra Lingner, Post-doc  
Sahar Pourebrahim, technician

**Key References for project**


**Own references (mainly 2009-2011):**


6.) Name: Falk, Christine, Prof. Dr.  
Institution: Institute of Transplant Immunology, IFB-Tx OE8889  
Dept. Leader: Prof. Dr. Dr. Christine Falk  
Telephone: ++(49) 511-532-9745  
Email: falk.christine@mh-hannover.de

Name of two Co-supervisors (from different departments):  
Prof. Dr. U. Baumann, Ped. Gastroenterology  
Dr. E. Jäckel, Gastroenterology

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: ☐ Name of student(s):  
Lecturer: X ☐; PhD Kommission: ☐ No, not yet: ☐

Animal experiments involved: ☐ yes X ☐ no

Research focus:  
We focus primarily on the following questions regarding the role of NK and T cells in liver Tx:  
The innate and the adaptive immune system – two immunological systems cooperating especially in the liver  
The liver represents the organ with the highest numbers of infiltrating Natural Killer cells (NK cells). As part of the innate immune system that comprises primarily NK cells (i.e. CD16/56dim or CD16/56bright NK cell subsets), monocytes and dendritic cells, NK cells can either support or even suppress specific T cell responses. T and B cells are part of the adaptive immune system due to their antigen-specific T or B cell receptors, respectively. Similar to viral or bacterial infections, alloreactivity is mediated by specific T cells recognizing allogeneic MHC class I or II molecules and activation via the T cell receptor (TCR) leads to execution of the most relevant effector mechanisms, i.e. cytotoxicity and cytokine/chemokine secretion. However, without guidance by the innate immune system, an adaptive immune response will remain ineffective and insufficient to completely eliminate pathogens or to reject allogeneic cells in solid organ or stem cell transplantation. For this purpose in guiding adaptive immune responses, the innate immune system transmits signals to the adaptive immune system. e. g. dendritic cells present pathogens to CD4+ T lymphocytes and activate CD8+ cytotoxic T cells (CTL) and NK cells support T cell activity by supplying cytokines. B lymphocytes are activated by CD4+ T cells, change into plasma B cells and produce specific antibodies. The adaptive immune system, therefore, is antigen specific also in the transplant setting: T cells carry their unique TCR recognizing allo-HLA molecules and B cells and plasma cells produce HLA-specific antibodies. The same mechanisms involved in immunity against pathogens are involved in allogeneic organ transplantation.  
Since the liver harbors a high proportion of NK cells, and NK T cells, it is particularly interesting to monitor both, innate and adaptive immune cells after LTx in the periphery and in liver biopsies if available. Elegant studies by have shown that up to three weeks after LTX but not renal Tx, for example, donor NK cells can be identified in the periphery of the recipient which differ phenotypically and functionally from normal peripheral recipient NK cells. Our own recent work on liver-resident NKL cells suggest that liver-infiltrating NK cells represent a unique NK subset with a different receptor equipment and, as a consequence, different effector functions regarding cytokine / chemokine secretion, in particular.
The composition of the cytokine / chemokine / growth factor milieu in serum / plasma after LTx represents an important source for potential biomarkers with respect to differences between various immune suppressive drugs like mTOR-inhibitors (i.e. Certican) vs. Calcineurin-Inhibitors (CNI). The differences are likely to result from the different modes of action since CNI target primarily NFAT-dependent molecules like the cytokines IL-2, IFN-g, IL-12p70 in immune cells while mTOR-inhibitors may also affect PI3-kinase signaling pathways in non-immune cells such as endothelial and parenchymal cells, for instance.

Therefore, one of the most important open questions in LTx are on one hand, the relevance of these donor NK cells and on the other hand, the influence of different immune suppressive drugs on NK cells, again liver-residing NK cells, in particular. Most comparative studies on the influence of immune suppressive drugs so far are focusing on T and B cells. Therefore, it is the aim of our proposal to include the different NK cell subsets into the immune monitoring in this multi center trial. In addition, the changes in the composition of cytokines, chemokines, growth factors and soluble liver factors between the treatment groups will be determined in order to define conditions of the peripheral microenvironment that support graft survival and function.

Specific project and methods applied:
Title: The role of NK cell and T cell subsets and the cytokine/chemokine microenvironment in paediatric liver transplantation
Aims: In the proposed project, the phenotype and function of NK cells and T cells in the perfusion solution and the peripheral blood of LTx patients after Liver Tx shall be investigated in parallel to the microenvironment in these compartments. Therefore, the peripheral blood of paediatric patients before and after liver Tx shall be analyzed with respect to the composition of the most prominent NK can T cell subsets. In addition, the presence of these cells in perfusion solution shall be tested in order to define their potential involvement in tolerizing mechanisms that characterize liver Tx much more than transplantation of other organs. The ultimate aims are the definition of parameters that correlate with the immunological status after LTx, i.e tolerance of the organ or rejection episodes.

Funding: DFG SFB738 project B8
For the quantification of peripheral lymphocyte subsets, peripheral blood within the first three weeks after LTx will be required. If available, liver biopsies would be ideal to determine the microenvironment in the liver after Tx and to define differences between the treatment groups that are related to the mechanism of action of these drugs: For the procedure of peripheral immune monitoring, peripheral blood (EDTA blood) from each patient within the first three weeks after LTx is investigated. In addition, perfusion solution directly after LTx shall also be analyzed with respect to the cellular composition and the microenvironment. Peripheral subpopulations of the following immune cells will be determined by flow cytometry using the 7 color LSR II FACS analyzer. The numbers of T and NK cells per µl peripheral blood will be determined using the CE certified True Count technique (BD, USA. In order to define the distribution if lymphocyte subsets, all other FACS panels (fist panels for the major subsets) will be analyzed with PBMC (peripheral blood mononuclear cells) after Ficoll separation. From each blood sample, at least 1-2 vials of PBMC will be frozen and stored in liquid nitrogen for further characterization in secondary panels.

Subpopulations of NK cells:
NK cells express an individual receptor repertoire that determines their major functions, cytotoxicity and cytokine production. Due to their selective expression of several markers two
major NK cells subsets can be identified: CD16+CD56dim comprising about 90% and CD16-CD56bright comprising about 10% of NK cells in peripheral blood. Remarkably, the composition of these two NK cell subsets is reversed in most organs, especially in the liver. In addition, we have identified a novel marker for NK cell infiltration into solid organs with the highest significance among all other 20 NK cells markers that are routinely incorporated into the experimental marker panels. Since CD16 binds to antibodies, antibody-mediated rejection may be accompanied by NK cell activation. Therefore, the Fcγ receptor CD16 is analyzed in combination with activation markers CD25 and CD69 and others. Further markers sets (secondary panels) comprise receptor panels such as killer-immunoglobin receptors (KIR), C-type-lectin receptors (CD94/NKG2A; NKG2D) natural cytotoxicity receptors (NCR like NKp30, NKp44, NKp46) as well as co receptors, CD244 (2B4), and DNAM-1.

Donor NK cells in the periphery of LTx recipients:
According to the HLA class I or II mismatch between LTx donor and recipient, donor-derived NK cells that have migrated our of the transplanted liver into the periphery of the recipient, can be identified by FACS staining of mismatch HLA alleles on donor or recipient cells. Due to the limited availability of HLA-specific antibodies, these studies will not be possible with all patients.

Subpopulations of T lymphocytes:
CD4+ and CD8+ cells (CD4+ T helper cells Th1/Th2/Th17; CD8+ cytotoxic T cells CTL ) will be determined as naïve (CD45RA) or memory T cells (CD45R0); regulatory T cells (Treg) will be monitored by intracellular staining of FoxP3 in combination with CD4 and CD25 as recommended by several international immune monitoring consortia. In addition, the classical activation markers CD25 and CD69 will be included and other markers for important T cell subsets such as IL7/ (CD127), and chemokine receptors, CCR5-7, CXCR1-4, for instance (secondary panels).

Quantification of the microenvironment in plasma and perfusion solution:
The microenvironment in serum / plasma and perfusion solution in paediatric patients after LTx can be determined using a multiplex technique measuring up to 60 parameters simultaneously in 50 µl serum or plasma, respectively. The addition, this approach will enable us to define whether complex networks of cytokines, chemokines, angiogenetic factors from non-invasive serum samples can be used for a discrimination of tolerant from non-tolerant patients. These cytokine / chemokine / growth factor panels comprise the most important cytokines, chemokines and proteins involved in angiogenesis and endothelial integrity. In addition, a novel liver pane including members of the fibroblast growth factor and angiopoietin families, hepatocyte growth factor and others will be included in order to combine the immune parameters with some liver function markers apart from transaminases.

Time schedule
1. Establishment of functional assays for NK cell function in peripheral blood and perfusion solution; multicolor flow cytometric analysis of T and NK cells comprising the most relevant subsets. Quantification of cytokines and chemokines in plasma and perfusion solution of patients with paediatric liver Tx patients (first 18 months).
2. identification of protein markers in plasma for the individual adjustment of immunosuppression after TLx (second 18 months)

Group Members:
Christine Neudörfl, PhD, POstDoc, Bernadette Müller PhD student, from 06/2012 PostDoc; Jana Keil, technician, Kerstin Daemen, technician. MAja Stevanovic-Meyer, technician

Zurück an: kruse.susanne@mh-hannover.de
Key References for project


Own references (mainly 2009-2011):


Zurück an: kruse.susanne@mh-hannover.de
Research focus:

Our research focuses on the signalling downstream to the p38 MAP kinase family. p38 MAPK isoforms are activated by pro-inflammatory cytokines, osmotic and chemical stress, and UV irradiation. MAPKAP kinases 2 (MK2) and 3 (MK3) are well characterised downstream targets of p38. MK2 and MK3 are indistinguishable in regard to p38-dependent regulation and activation as well as substrate specificity, but MK2 has predominant physiological relevance due to its relative abundance.

MK2/3 has been implicated in post transcriptional regulation of inflammatory gene expression mediated by AU-rich elements. Consistently, MK2/3 deficiency is characterized by enhanced resistance in different murine models of inflammation. In addition a role for p38 and MK2/3 in cytoskeletal dynamics is evident by the reduced migratory capacity of p38- and MK2/3-deficient cells. MK2/3 were shown to phosphorylate and regulate multiple regulators of actin cytoskeleton which include Hsp25/27, LIM kinase-1, Vimentin, CapZ-interacting protein (CapZIP), and Leukocyte specific protein 1 (LSP1). We could recently identify type 1 simple epithelial keratins as MK2/3 substrates and several studies have shown the involvement of the p38 pathway in microtubule reorganisation, but the mechanism remains unknown. Data suggest active regulatory roles for the pathway also in angiogenesis, oncogenesis, ischemia reperfusion injury, cellcycle regulation, wound healing and immune cell migration.

Specific project and methods applied:

**Title:** Regulation of cytoskeletal dynamics and cell motility by the MAPKAP kinases MK2 and MK3

**Aims:** Characterization of novel cytoskeletal substrates of MK2/3 and defining the underlying signaling events contributing to actin/microtubule reorganization and cell migration

**Funding:** Landesmittel/later SFB1065

p38/MK2/3 activation strongly influences cytoskeletal dynamics, cell morphology and motility. The known substrates of MK2/p38 could not completely explain the role played by the pathway in actin-microtubule regulation. Yeast two hybrid and more recent phospho-proteomics studies in our lab identified novel MK2/3 -interacting proteins and substrates critical for cytoskeletal dynamics. The novel putative substrates include MAP1A-LC2, KAP3 and other guanine nucleotide exchange factors and actin binding proteins. The new putative substrates of MK2/3 identified in our preliminary studies could help develop a model where p38 and the MKs act as master kinases regulating actin, microtubule and keratin based active cellular events. The specific aspects of the project include:

**A. Biochemical characterisation of putative substrates identified in Y2H and phosphoproteomics:** The identified downstream targets will be cloned and interactions analysed in mammalian cell culture systems. In vitro kinase assays and mutational analysis would be done to identify direct substrates and phosphorylation sites. Antibodies for phosphorylation sites should be generated if not available.
B. Analysis of roles of newly identified and known substrates: Quantitative scratch wound healing assay as well as transwell migration assays would be used to characterise the role of MK2 substrates in cell migration. Dominant negative and active mutants (of Rho GTPases), phospho-mimicking and phospho-dead mutants of substrates, inhibitor and knock-down approaches will be used. Similar functional assays for cell polarization, chemotaxis and endothelial permeability will be performed. Localisation and signal dependent reorganisation of cytoskeleton and co-localization of the substrates will be studied using confocal microscopy.

C. MK2/3 knock out cells as a model to study the role of p38/MK pathway in cytoskeleton remodelling: Since there are multiple cell type specific substrates which contributes to MK2 mediated actin rearrangement, different cell types from MK2/3 deficient mice will be analyzed in above mentioned assays for active cell movement. The mice model could also be used to study the in vivo role of MK2/3 in cell motility, fibrosis and cardiac remodeling.

Time schedule
1. Project parts A and B will be started in the first year
2. Project part A will be finished in the second year and part C will be started.
3. In the third year, parts B and C will be finished.

Group Members:
Matthias Gaestel, Group leader; Alexey Kotlyarov, Senior Scientist and co-group leader; Mohammed Tehrani, PhD student; Frank Brand, PhD student; Christopher Tiedje, Post Doc; Manoj B. Menon, Post Doc; Natalia Ronkina, Post Doc; Kathrin Laß, Lab manager; Tatiana Yakovleva, Technician; Juri Lafera, Technician.

Key References for project

Own references (mainly 2009-2011):
Name: Prof. Faikah Güler  
Institution: Dept. of Nephrology 
Dept. Leader: Prof. Dr. Haller  
Telephone: +49 (511) 532-3722, +1761 532 8466  
Email: gueler.faikah@mh-hannover.de

Name of two Co-supervisors (from different departments):
Prof. Hilfiker-Kleiner Experimental Cardiology 
Prof. Wacker Dept. of Interventional and Diagnostic Radiology

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: ☐ Name of student(s):
Lecturer: ☐; PhD Kommission: ☐; No, not yet: X (I would like to give lectures)

Animal experiments involved:  ☑ yes ☐ no

Research focus: Hypoxia induced kidney transplant rejection in mice

Specific project and methods applied:
Title: Role of PKC alpha and beta deficiency in hypoxia induced kidney transplant rejection in mice

Aims: We have shown that a protein kinase C (PKC) alpha and beta inhibitor prevents inflammation and apoptosis in acute kidney injury (AKI) via different signaling mechanisms (TNF-alpha and TGF-beta signaling). In this project, we want to investigate whether PKC alpha or beta isoforms are involved in acute kidney transplant rejection in mice as well as in progressive interstitial fibrosis and tubular atrophy which is a correlate for chronic rejection. Knock out mice for PKC alpha and for PKC beta isoforms as kidney donors will be used for the experiments. Functional measurements for renal function and perfusion and tissue analysis (BANFF classification for rejection, histology and immunohistology for leukocyte infiltration) as well as western blotting (MAP kinase activation), qPCR (up-regulation of adhesion molecules), FACS analysis (cytokine expression: KC, MCP-1, IL-6) will be performed.

Funding: BMBF (Professorinnen Programm)

Previously, we have shown that a PKC alpha / beta isoforms inhibitor prevents acute kidney injury due to transient ischemia reperfusion injury fibrosis in mice. Mechanisms were activation of TNF-alpha and TGF-beta dependent signaling cascades. By using knock out mice it is not clear whether the beneficial effects of PKC deficiency is due to signaling defects in the infiltrating leukocytes or are related to local deficiency in renal cells (such as tubular epithelial cells). In the transplant model PKC deficient grafts can be transplanted in WT recipients so that the different contributing factors can be clearly identified. Just recently functional MRI sequences have been established in collaboration by our group with Dr. Hüper (radiology, MHH) and Dr. Meier (MRI facility, MHH-ZTL) to measure renal perfusion and diffusion defects of the mouse kidney. This will give us the opportunity to measure edema formation due to inflammation and perfusion defects as well as tissue diffusion.

A. Kidney tx will be performed in 3 groups: PKC alpha -/-, PKC beta -/- and WT controls will be compared in terms of survival and renal function
B. Functional MRI measurements will show differences in edema formation due to acute kidney injury, reduction of diffusion by leukocyte infiltration in the early phase (i.e. 3 days after ktx) and decrease of perfusion.

C. Inulin clearance measurements to estimate glomerular filtration rate have to be performed on additional mice. Furthermore, histological work up will be performed to investigate type of infiltrating cells (leukocyte sub-types lymphocytes CD4, CD8, macrophages F4/80, neutrophils GR-1, cleaved caspase-3, TUNEL stain). Systemic cytokine release will be measured by FACS based bead assay (TNF-alpha, IL-6, MCP-1, KC).

D. Long term evaluation after 1, 2 and 3 months after ktx will be performed and expression of pro-fibrotic proteins (CTGF, fibronectin, collagen 4) will be studied.

**Time schedule**

**Work package 1:**
1.-2. months: Training of basic skills (duration varies depending of the prior experience of the student): handling of the mice, basics in mouse surgery ischemia reperfusion injury, kidney transplantation, renal nephrectomy (surgical supervisor is Dr. Rong). Training course of the central animal facility. In addition training of histological work-up: paraffin embedding, cutting with a microtome of paraffin embedded tissue as well as cryosections, staining procedures, cytokine measurements,

**Work package 2**
12-16 months: Surgical experiments, survival curves, IR as well as kidney tx experiments for acute kidney damage and rejection: Inulin / PAH clearance measurements. Mice will be sacrificed at different time points (d1, d6, d28). Work up of the material by FACS bead assay, histology, western blotting and qPCR.

**Work package 3:**
6 months: functional MRI for the different groups – each group needs to contain 6-8 mice. 4 mice a day can be evaluated by MRI. The most interesting time points will be evaluated to get additional information on edema formation and perfusion reduction.

**Potential further work package 4:**
The model for progressive interstitial fibrosis and tubular atrophy which is the correlate for chronic rejection can be evaluated by C4d staining, complement activation and antibody formation.

**Potential of the project in terms of publications:**
Since the PKC alpha/beta inhibitor showed excellent effects to prevent inflammation and fibrosis in the previous IR injury project the investigation of knock out mice will most likely very successful in terms of publishable results. The IR injury model can be published as well as a further manuscript on acute transplant rejection is possible. Furthermore, the late phase of tx rejection due to progressive interstitial fibrosis will be evaluated and might lead to another manuscript depending on the ongoing project further testings of anti-inflammatory compounds can be added to the project in the established models. My group has a big expertise in drug testing for preventing IR injury currently we have several compounds in early testing. Here also a student can be involved.

**Group Members:**
StrukMed Student; Nele Rüskamp, MD student; Franziska Zylka, Nils Hanke
PhD: Dr. Nelli Shushakova
Surgeon: Dr. Rong Song (Microsurgery in mice)
Technician: Herle Chlebusch, Hayet Richie

Zurück an: kruse.susanne@mh-hannover.de
Cooperation partner: Dr. Hüper (Radiology), Dr. Meier (ZTL MRI facility)

Key references for project


Additional submitted manuscripts related to the project


Own references (mainly 2009-2012):

1. Song Rong1,2, Alfor G. Lewis3, Uta Kunter4, Hermann Haller1, Faikah Gueler. A knotless Technique for Kidney Transplantation in the Mous. Accepted in Transplantation 2012


Zurück an: kruse.susanne@mh-hannover.de
9.) Name: PD Dr. med. A. Heim  
Institution: Dept. of Virology  
Dept. Leader: Prof. Dr. T. F. Schulz  
Telephone: ++(49) 511-532-4311  
Email: heim.albert@mh-hannover.de  
Name of two Co-supervisors (from different departments):  

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: X  Name of student(s): Fabienne Rehren, Elena Lam  
Lecturer: X;  PhD Kommission: ☐;  No, not yet: X  

Animal experiments involved: ☐ yes  X no  

Research focus:  
Our research focuses on virulence and tissue/organotropism of different human adenovirus types. Our previous studies have analyzed the phylogenetic relationship of adenovirus types focusing on determinants responsible for either interaction with the immune system or interaction with the host cell. This included a detailed analysis of the three main determinants responsible for binding to the host cells' primary and secondary receptor for adenoviruses (fiber knob: gamma determinant, penton base: delta determinant), discovery and complete genomic sequencing of a new adenovirus type (HAdV-D53) and complete genomic sequencing of another highly virulent type (HAdV-A31). A detailed mutagenesis of the secondary receptor binding determinant was performed including molecular modeling to establish the significance of conserved motifs in this determinant. Additional research focused on naturally occurring recombinant viruses (between adenovirus types) and their altered virulence and tropism. Recently we have shown that tropism and virulence of an adenovirus type can be changed completely after recombination events have substituted these determinants with sequences from highly pathogenic adenovirus types (in case of HAdV-D53).  
Additional studies focused on adenovirus types associated with severe disseminated infections in highly immunosuppressed patients, for this purpose virus load diagnostics and molecular typing were established.  
Human adenoviruses are highly specific for their human host, thus standard rodent model systems are not available or not appropriate. Therefore, cell- and tissue culture systems of human and porcine origin were developed in order to quantify binding and replication efficacy of different adenovirus types on differentiated cells as well as techniques to establish the specific influence of different adenovirus types on the host cell gene expression including e.g. cytokine response. Special focus of the project is now the pneumonia associated human adenovirus type 14a (=14p1). Previously, only two of 65 adenovirus types caused pneumonia and acute respiratory distress syndrome (ARDS) with high mortality but almost exclusively in military trainees. Therefore, a combination of lower airway tropism, droplet infection mode due to crowding and physical training were suspected to be essential for disease manifestation. However, a new adenovirus subtype 14a has appeared recently which caused pneumonia and ARDS in a civilian setting without specific risk factors. This Adenovirus type has been isolated in the US and recently also in Ireland. This clinical isolate is available to us and should be studied in this project.  

Specific project and methods applied:  
Title: Virulence factors of Pneumotropic Adenoviruses Type 14
**Aims:** In the proposed project the interaction of the newly discovered human adenovirus type 14a with its host cells (airway epithelium, alveolar cells) should be analyzed in comparison to non- and less- pneumotropic adenovirus types (2, 3 and 4).

**Funding:** LOM/DFG (planned)

All pneumotropic human adenoviruses as well as non pneumotropic adenovirus types are in our collection. Polarized airway/liquid culture of airway cells has been established both of human and porcine origin. Although porcine cells were found to be permissive for human adenoviruses, they were found not to be appropriate for studies with type 14a. Preliminary studies indicated that this type infects human airway epithelium cells more efficiently from the apical surface and infectious virus progeny will also be released to the apical surface, thus promoting spread of the virus through the airways. In this human cell model the following topics should be addressed:

A. Interference of adenovirus infection with cellular gene expression.

Previous work has suggested that cytokine induction by human adenoviruses is adenovirus type dependent and cell type dependent. So far, no research project has studied the effects of adenovirus replication on primary human bronchial epithelium and alveolar cells. Moreover, a direct comparison of cellular gene induction by pneumotropic and non pneumotropic adenovirus types has never been performed before. Techniques to address these topics are established (mRNA microarray, quantitative PCR) and should be easily applied.

B. Adenovirus binding to cellular receptors

As the receptor use of adenovirus type 14a has not been completely elucidated, binding of pneumotropic viruses to potential receptors (Desmoglein2, CAR, CD46, as primary and αvβx integrins as secondary receptors) should be studied with help of blocking, knock down and overexpression experiments on standard cell lines. Results should be confirmed on polarized airway epithelium cells.

C. Molecular studies on adenoviral virulence factors.

Adenoviral genes coding for structural and non structural proteins should be studied. Previous research on A549 cells showed that structural proteins (as e.g. the penton protein) can be directly cytotoxic. Therefore, structural genes of pneumotropic and non pneumotropic adenovirus types should be expressed in E. coli and tested for interaction with primary human lung cells (bronchial epithelium and alveolar cells). Adenoviral non structural proteins interacting with the (innate) immune system are mainly coded by the E3 region, which is highly divergent between adenovirus types. Genes of pneumotropic and non pneumotropic types should be cloned and expressed in human lung cells as well as established cell lines to study their effects on expression of cytokines and cytokine receptors as well as other cellular factors interacting with the immune system (e.g. MHC-I expression).

D. Confirmation with recombinant adenovirus types

Identified virulence factors (see "C") and binding sites for cellular receptor (fiber knob, penton base) will be tested for their significance on the pneumotropic phenotype (efficient apical infection and release) of adenovirus type 14a with help of recombinant adenoviruses generated with help of bacterial artificial chromosomes (BAC) technology. BAC cloning will be performed in collaboration with the group of Prof. Ehrardt (Witten-Herdecke) as a project of the planned "adenovirus research group".
**Time schedule**
Topic A should be completed within the first 6 months. In parallel first steps for topic C (cloning) can be started. Between months 6 and 18 experiments in differentiated cells for topics B and C should be performed. Topic D can be studied (depending on the preliminary results of C) during the second half of the project.

**Group Members:**
Elena Lam, post doc; Dominik Markel Dr. med. student; Mirja Ramke Dr. med. student
Gabriele Harste, technician

**Key References for project**


Short JJ, Pereboev AV, Kawakami Y, Vasu C, Holterman MJ and Curiel DT. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 2004;322:349-59


**Own references (mainly 2009-2011):**


10.) Name: Prof. Dr. Andreas Kispert  
Institution: Dept. of Molecular Biology  
Dept. Leader: Prof. Dr. Achim Gossler  
Telephone: ++(49) 511-532-4017  
Email kispert.andreas@mh-hannover.de

Name of two Co-supervisors (from different departments): ?? to be named after selection of candidates

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: yes  
Name of student(s): Reena Singh, Rannar Airik, Franziska Greulich  
Lecturer: yes.

Animal experiments involved: ☐ yes

Research focus:  
Our research focuses on the genetic control mechanisms governing vertebrate development using the mouse as a model organism. More specifically, we are interested in gaining a better understanding of the cellular and molecular processes crucial in the development of mesodermally derived organs including the heart, kidney and ureter, and the inner ear. This work also aims to better understand congenital human diseases including valvular-septal defects of the heart, anomalies of the urinary tract and hearing loss by establishing mouse models for these disorders and deciphering the molecular changes associated with them. In the long run, this may provide means to reinitiate developmental programs for regenerative approaches in human diseases.

We are using all techniques of modern cell and molecular biology in addition to embryological manipulations in the mouse as well as transgenics and targeted gene disruption to gain information on the mode of action of genes in vivo and in vitro.

A major focus of our molecular work lies in the analysis of the role of transcription factors of the T-box (Tbx) gene family in organogenesis.

Specific project and methods applied:  

Title: Molecular analysis of Tbx2/Tbx3 in kidney collecting duct development in the mouse  
Aims: In the proposed project the function of Tbx2/Tbx3 in the development of the collecting duct system of the kidney shall be analyzed in the mouse.  
Funding: Haus/DFG

Tbx2 and Tbx3 are two closely related members of the Tbx gene family of transcription factors that play important (often redundant) roles during embryonic organ formation in vertebrates. We have previously shown the requirement for these genes in liver, lung and heart development, and plan now to analyze the functional implication of these genes in the development of the kidney and ureter in the mouse. Our preliminary work has shown that both genes are expressed in the ureteric epithelium, in the ureteric mesenchyme and in the mesenchyme of the kidney proper including the glomerulus. We have floxed alleles of both Tbx2 and Tbx3 and suitable cre lines available to analyze the functional requirement of these genes in the various compartments of the kidney and ureter.
A. Analysis of Tbx2 and Tbx3 expression during urogenital development both on the level of mRNA and protein in whole mount specimens and on sections to gain a high resolution image of unique and overlapping expression of both genes.

B. Phenotypic analysis of Tbx2 and Tbx3 single mutants as well as Tbx2/Tbx3 double mutants by conditional approaches in the mouse. Floxed alleles of Tbx2 and Tbx3 shall be crossed with a specific cre line that mediates recombination in the ureteric epithelium (Pax2cre) and the phenotypic consequences analyzed during development. For this purpose, urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

C. Mis/overexpression of Tbx2 in the ureteric epithelium using an available HprtTbx2 knock-in allele and the Pax2cre line. Urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

D. Identification of target genes of Tbx2 and Tbx3 in the kidney and ureter using microarray and CHIP analyses of embryonic tissues, and subsequent evaluation as candidates by expression and/or functional analyses.

**Time schedule**
1. Year 1-year2: A, B
2. Year 2-Year3: B, C
3. Year 3: D
   (4. Year 4: D)

**Group Members:**
<5> postdocs;
<6>, PhD students;
<2>, Bachelor students;
<1>, technician as of May 1st 2012

**Key References for project**


Zurück an: kruse.susanne@mh-hannover.de
Own references (mainly 2009-2011):
Landgraf, K., Bollig, F., Trowe, M.-O., Besenbeck, B., Ebert, C., Kruspe, D., Kispert, A., Hänel, F.,

Zurück an: kruse.susanne@mh-hannover.de


Our research focuses on the genetic control mechanisms governing vertebrate development using the mouse as a model organism. More specifically, we are interested in gaining a better understanding of the cellular and molecular processes crucial in the development of mesodermally derived organs including the heart, kidney and ureter, and the inner ear. This work also aims to better understand congenital human diseases including valvular-septal defects of the heart, anomalies of the urinary tract and hearing loss by establishing mouse models for these disorders and deciphering the molecular changes associated with them. In the long run, this may provide means to reinitiate developmental programs for regenerative approaches in human diseases.

We are using all techniques of modern cell and molecular biology in addition to embryological manipulations in the mouse as well as transgenics and targeted gene disruption to gain information on the mode of action of genes in vivo and in vitro.

A major focus of our molecular work lies in the analysis of the role of transcription factors of the T-box (Tbx) gene family in organogenesis.

Specific project and methods applied:
**Title:** Molecular analysis of Tbx2/Tbx3 in the development of the mesenchymal compartments of the kidney and ureter in the mouse

**Aims:** In the proposed project the function of Tbx2/Tbx3 in the development of the mesenchymal compartments of the kidney and ureter shall be analyzed in the mouse.

**Funding:** DFG

Tbx2 and Tbx3 are two closely related members of the Tbx gene family of transcription factors that play important (often redundant) roles during embryonic organ formation in vertebrates. We have previously shown the requirement for these genes in liver, lung and heart development, and plan now to analyze the functional implication of these genes in the development of the kidney and ureter in the mouse. Our preliminary work has shown that both genes are expressed in the ureteric epithelium, in the ureteric mesenchyme and in the mesenchyme of the kidney proper including the glomerulus. We have floxed alleles of both Tbx2 and Tbx3 and suitable cre lines available to analyze the functional requirement of these genes in the various compartments of the kidney and ureter.
A. Analysis of Tbx2 and Tbx3 expression during urogenital development both on the level of mRNA and protein in whole mount specimens and on sections to gain a high resolution image of unique and overlapping expression of both genes.

B. Phenotypic analysis of Tbx2 and Tbx3 single mutants as well as Tbx2/Tbx3 double mutants in the metanephric and ureteric mesenchyme by conditional approaches in the mouse. Floxed alleles of Tbx2 and Tbx3 shall be crossed with specific cre lines that mediate recombination in the ureteric mesenchyme (Tbx18cre) and the metanephric mesenchyme (Six2cre) and the phenotypic consequences analyzed during development. For this purpose, urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

C. Mis/overexpression of Tbx2 in the metanephric and ureteric mesenchyme using an available HprtTbx2 knock-in allele and the Six2cre and Tbx18cre lines. Urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

D. Identification of target genes of Tbx2 and Tbx3 in the kidney and ureter using microarray and ChIP analyses of embryonic tissues (kidneys and ureters).

**Time schedule**
1. Year 1-year2: A, B
2. Year 2-Year3: B, C
3. Year 3: D
4. Year 4: D

**Group Members:**
<5> postdocs;
<6>, PhD students;
<2>, Bachelor students;
<1>, technician as of May 1st 2012

**Key References for project**

**Own references (mainly 2009-2011):**


dimensional architecture of the atrioventricular conduction axis of the mouse heart. Circ. Res 107, 728-736.


Research focus:
Our research focuses on the genetic control mechanisms governing vertebrate development using the mouse as a model organism. More specifically, we are interested in gaining a better understanding of the cellular and molecular processes crucial in the development of mesodermally derived organs including the heart, kidney and ureter, and the inner ear. This work also aims to better understand congenital human diseases including valvular-septal defects of the heart, anomalies of the urinary tract and hearing loss by establishing mouse models for these disorders and deciphering the molecular changes associated with them. In the long run, this may provide means to reinitiate developmental programs for regenerative approaches in human diseases.

We are using all techniques of modern cell and molecular biology in addition to embryological manipulations in the mouse as well as transgenics and targeted gene disruption to gain information on the mode of action of genes in vivo and in vitro.

A major focus of our molecular work lies in the analysis of the role of transcription factors of the T-box (Tbx) gene family in organogenesis.

Specific project and methods applied:
Title: Signaling pathways governing smooth muscle differentiation of the ureteric mesenchyme in the mouse
Aims: In the proposed project the function of Pdgfr signaling as well as intracellular signaling modules in smooth muscle differentiation of the ureteric mesenchyme shall be analyzed.
Funding: DFG

The urinary system is a multi-component entity, whose primary functions are the maintenance of body homoeostasis by controlling the water and ionic balance of the blood and the excretion of excess water, solutes, and waste products. Rather than being a simple, passive tubular outlet of the pelvis, the ureter represents a pivotal connection between the upper (the kidneys) and lower urinary system (the bladder and the urethra). After filling the renal pelvis with urine, the upper portion of the ureter undergoes unidirectional peristaltic contractions, triggered by pacemaker cells, to propel the urine down to the bladder whilst preventing any reflux or efflux at the same time. The crucial importance of the ureter for renal function is dramatically reflected by acquired and inherited defects that interfere with the efficient removal of the urine from the renal pelvis. Any kind of anatomical or functional obstruction along the ureter or at its junctions will result in fluid pressure-mediated dilation of the tubular system proximal to the side of constriction (hydroureter and hydronephrosis).
In contrast to other organs such as the kidney, our knowledge of the genetic control of ureterogenesis has been limited. However, recent findings suggest that formation of the ureter relies on a multi-step developmental program that is characterized by the interaction of different mesenchymal and epithelial cell lineages of the early metanephric field. Our own experiments have revealed the importance of the ureteric mesenchyme and the T-box transcription factor Tbx18 therein for this process. In Tbx18−/− mice, the ureteric mesenchyme disperses and remains undifferentiated leading to hydroureter formation by functional obstruction. Other work of the lab has uncovered the crucial relevance of Sox9, Bmp4, Smad4, Shh and canonical Wnt signaling for smooth muscle differentiation of the ureteric mesenchyme. Our preliminary work has shown that loss of Pdgfra signaling also results in hydroureter formation due to a lack or reduction of smooth muscle cell differentiation. Hence, we want to define the role of Pdgfr-signaling and its downstream modules in differentiation of the ureteric mesenchyme by genetic loss- and gain-of-function approaches in the mouse as well as by pharmaceutical experiments in vitro.

A. The functional significance of Pdgfra signaling in differentiation of ureteric mesenchyme shall be studied by a ureter-specific loss-of-function approach. A floxed allele of Pdgfra shall be crossed with a specific cre line that mediates recombination in the ureteric mesenchyme (Tbx18cre) and the phenotypic consequences analyzed during development. For this purpose, urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level (in situ hybridization of candidate genes, microarrays) for changes of tissue histology, proliferation/apoptosis, and gene expression.

B. The functional significance of Pdgfra signaling in differentiation of ureteric mesenchyme shall be studied by a ureter-specific gain-of-function approach. An inducible allele of an activated form of Pdgfra shall be generated and crossed with a specific cre line that mediates recombination in the ureteric mesenchyme (Tbx18cre) and the phenotypic consequences analyzed during development. For this purpose, urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

C. The functional significance of Pdgfrb signaling in differentiation of ureteric mesenchyme shall be studied by a ureter-specific loss-of-function approach. A floxed allele of Pdgfrb that is already in house shall be crossed with a specific cre line that mediates recombination in the ureteric mesenchyme (Tbx18cre) and the phenotypic consequences analyzed during development. For this purpose, urogenital systems shall be isolated from embryonic stages and kidney and ureters analyzed on the histological, immunohistochemical and molecular level for changes of tissue histology, proliferation/apoptosis, and marker gene expression.

D. The relevance of downstream signaling modules for smooth muscle differentiation shall be analyzed by pharmaceutical inhibition experiments in cultures of kidney rudiments. For that specific inhibitors and combinations of inhibitors, respectively, shall be applied to ureter cultures and the consequences for smooth muscle cell differentiation analyzed by immunofluorescence.

**Time schedule**
1. Year 1-Year2: A, B
2. Year 2-Year3: B, C
3. Year 3: D
(4. Year 4: D)

**Group Members:**

Zurück an: kruse.susanne@mh-hannover.de
<5> postdocs;
<6>, PhD student;
<2>, Bachelor students;
<1>, technician as of May 1st 2012

Key References for project

Own references (mainly 2009-2011):

Zurück an: kruse.susanne@mh-hannover.de


Kispert, A. (2012). No muscle for a damaged heart: Thymosin beta 4 treatment after myocardial...


13.) Name: Dr. J.H. Klusmann  
Institution: Dept. of Pediatric Hematology and Oncology  
Dept. Leader: Prof. Dr. D. Reinhardt  
Telephone: ++(49) 511-532-3252  
Email Klusmann.jan-henning@mh-hannover.de  
Name of two Co-supervisors (from different departments): Prof. T. Thum

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: X Name of student(s): Stephan Emmrich and Aliaksandra Maroz  
Lecturer: □; PhD Kommission: □; No, not yet: □

Animal experiments involved: X yes □ no

Research focus:  
Our research focuses on the genetic control mechanisms underlying hematopoietic development as well as leukemogenesis in the murine and human system (Li et al, Nat Genet 2005; Klusmann et al, Genes Dev 2010b). Recent advances in the exploration of the transcriptome revealed non-coding RNAs (ncRNAs) as important genomic regulators. MicroRNAs (miRNAs) are ∼20-24nt RNAs that negatively regulate eukaryotic gene expression at the post-transcriptional level and reflect the best studied subgroup among regulatory ncRNAs. They control genetic networks with key cellular functions and are implicated in the development of cancer (Esque-la-Kerscher et al., Nat Reviews Cancer 2006; Emmrich & Pützer, Cell Cycle 2010). About 50% of the annotated human miRNAs map within fragile regions of chromosomes, which are associated with various human cancers (Calin et al., PNAS 2004). Some miRNAs are deregulated in cancer and can affect cellular transformation, carcinogenesis and metastasis acting either as oncogenes or tumor suppressors, depending on their target genes (Klusmann et al, Genes Dev 2010a).  
Acute myeloid leukemias reflect the largest group of malignancies during childhood, whereas recurrent chromosomal translocations in acute myeloid leukemia (AML) frequently affect the mixed-lineage leukemia gene (MLL), the master hematopoietic transcription factor RUNX1 (t[8;21]; AML-ETO) or its cofactor (inv[16]; CBFβ-MYH11) or the retinoic acid receptor (t[15;17]; PML-RARA)(von Neuhoff et al, JCO 2010). Despite the growing knowledge about the molecular consequences of these fusion proteins, the precise pathomechanisms of leukemia are poorly understood. In particular, those translocations can be detected in blood spots of neonates, which progress to leukemia after a latency of up to 14 years in only a small fraction of cases (~0.1-1%). This suggests that additional genetic events have to occur in conjunction with those recurrent translocations.  
To identify putative cooperating factors in AML, we conducted three complementing high-throughput screening approaches: First, a global gene expression profile in more than 190 cytogenetically defined patient samples in a joint effort with the Erasmus MC, Rotterdam. Second, we perform a robot-assisted functional screening by applying a complete miRNA precursor (pre-miR) library and, third, an inhibitor (Anti-miR) library in four AML cell lines (representing MLL-rearranged, t[8;21], t[15;17] leukemias).  
In this project we will functionally characterize candidate miRNAs, with the aim to implement those ncRNAs in regulatory circuits of hematopoiesis and in novel concepts of leukemogenesis.
Specific project and methods applied:

Title: High-Throughput functional characterization of miRNAs in cytogenetically defined AML subgroups

Aims: The project is focused on the functional and molecular characterization of candidate miRNAs, identified by three complementing high-throughput screening approaches.

Funding: Other

Previously, we conducted a global gene expression profiling for mRNAs and miRNAs in cytogenetically characterized AML subgroups (MLL-rearranged, t[8;2], t[15;17], inv[16]). Furthermore a functional screening with pre- and anti-miRs was carried out in cell lines resembling the karyotypes, red out by a luminescent cell viability test. We compiled candidate lists of putative tumor suppressor or oncogenic miRNAs and established a streamlined cloning protocol to generate more than 50 lentiviral constructs. Moreover we optimized a meso-scale phenotyping strategy to characterize up to 10 constructs in parallel, which includes testing cell viability, cell growth, self-renewal, morphology, response to DNA-damage induction by Doxorubicin, to clinically applied cytostatics (cytarabin, etoposid) and to small compound-induced differentiation. The characterization pipeline was already applied to a batch of 20 candidates significantly deregulated in each of the three AML subgroups and yielded two hits displaying strong phenotypes. These two miRNAs (miR-139 and miR-582) markedly reduce cell proliferation and self-renewal in two t(8;21) cell lines and sensitize against doxorubicin and cytarabin by enhancing apoptosis and G1-arrest. Moreover, transcription of miR-139 can be induced by histone deacetylase inhibitor (HDACi) treatment.

The specific aims of this project are:

1. Validation of candidate miRNAs using a low-throughput/ high-output strategy
   - After completing the streamlined cloning of the full candidate list into our optimized vector system, a functional characterization of miRNAs in the respective cell lines will be performed using the meso-scale phenotyping strategy to characterize up to 10 constructs in parallel as described above.
   - The candidate list contains many potential pro-leukemogenic miRNAs, which are highly upregulated in the primary AML blasts and leukemic cell lines. When applying anti-miRs we observe a proliferation arrest, while the pre-miR did not show an inverse effect. This suggests that the endogenous miRNA level has passed a certain threshold and additional overexpression does not result in measurable effects. To gain further insight into the function of these miRNA, we take advantage of a previously reported approach using locked nucleic acid antisense oligonucleotides (LNA-ASOs), which target only the 8nt long miRNA seed (Obad et al, Nat Genet 2011). A transient transfection protocol was optimized. Tiny LNAs against miR-99/100, let-7 and miR-125 will be designed to validate the method. Afterwards, the effect of miRNA-knockdown will be assessed in a 4 day time course using the same methods and cell lines as described above.
   - Validated hits with a reproducible phenotype will be assessed in primary patient blasts and the molecular mechanism of single miRNAs will be investigated according to the strategy outlined in specific aim 2.

2. In depth characterization of previously validated candidate miRNAs
   The expression profiling and subsequent functional investigations (as outlined in specific aim 2) yielded two strong candidates (miR-139 and miR-582) for the t(8;21) subgroup both acting as tumor suppressor microRNAs.
   - We already performed a qPCR and SILAC-based miRNA target gene profiling yielding several promising candidates. These genes need to be validated as genuine targets via Western Blot and luciferase assays.
Findings in the cell lines will be investigated in primary human samples. An inducible state of the art lentiviral expression system will be applied to introduce the miRNAs in cord blood CD34⁺-hematopoietic stem and progenitor cells and leukemic blasts. We will use colony-forming assays with serial replating and liquid culture systems.

To evaluate the function of those miRNAs in vivo, we will transduce t(8;21) cell lines in vitro and transplant them into irradiated immunodeficient NSG mice. Additionally we will cotransduce fetal liver cells with the AML1-ETO fusion protein and the miRNA constructs. This setting will reveal, whether tumor suppressor miRNA overexpression can impair leukemogenesis in vivo.

**Time schedule**

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<td>Functional characterization of miRNA candidates in cell lines by tiny LNA knock-down</td>
<td>Cotransduction of AML1/ETO and miRNA constructs into murine fetal liver cells for bone marrow transplantation</td>
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**Group Members:**

Jan-Henning Klusmann; Group Leader  
Stephan Emmrich, PhD student;  
Aliaksandra Maroz, PhD student;  
Erik Pittermann, PhD student;  
Lena Stachorski, PhD student;  
Felix Engeland, MD student  
Alexandra Streltsov, MD student  
Kerstin Henke, MD student  
Mareike Rasche, MD student  
Jennifer Schöning, technician  
Anna-Lisa Queißer, technician  
Tolga Hazerli, technician
Key References for project


Own references (mainly 2009-2011):


14.) Name: Dr. Andrea Kröger  
Institution: Helmholtz Centre for Infection Research, AG Innate Immunity and Infection  
Dept. Leader: Dr. Hansjörg Hauser  
Telephone: +(49) 531-6181-5060  
Email: andrea.kroeger@helmholtz-hzi.de  
Name of two Co-supervisors (from different departments): S. Weiss, B. Sodeik

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: ☐ Name of student(s):  
Lecturer: ; PhD Kommission: ☐; No, not yet: x

Animal experiments involved: x yes ☐ no

Research focus:  
The immune system serves to protect the host by constantly monitoring the body for foreign agents and to identify and eliminate transformed or infected cells. The interferon (IFN) system plays a central role in this process of cancer immunoediting. Our research focuses on investigating the role of Interferon Regulatory Factor -1 (IRF-1) in immune regulatory function in tumor and metastasis development.

IRF-1 is involved in the regulation of the IFN system, and serves as a link between the innate and adaptive immunity. IRF-1 is a transcription factor, strongly induced by infections, IFN-γ and proinflammatory cytokines such as TNF-α, IL-1 and IL-6. Studies from gene knock-out mice revealed IRF-1 to favor T cell differentiation and NK cell development. The functions of IRF-1 are diverse and play crucial roles in a variety of biological processes such as inflammation, antiviral response, immune regulation and tumor suppression. In solid and lung metastasis models we have shown IRF-1 expression to induce tumor-specific immune responses. Although the same tumor cells are injected in different mouse tumor models the nature of immune response varies. The aim of the project is to understand the immune regulatory functions of IRF-1 expression in tumor cells and in the microenvironment of the growing tumor cells. Tumor cells and tumor stroma interact with each other by producing various mediators and cytokines which result in the infiltration and activation of immune cells. The project will focus on the regulatory mechanisms which drive the interaction between tumor cells and the tumor microenvironment and the elucidation of the function of IRF-1 in the inhibition of tumor development.

Specific project and methods applied:  
Title: Role of Interferon Regulatory Factor-1 tumor suppression and immune surveillance  
Aims: The project aims to investigate the impact of IRF-1 in tumor cell elimination. The first part of the project will involve investigating cell populations with tumor suppressive functions in a liver metastasis model. A second crucial aspect of the project involves investigating the impact of IRF-1 on regulatory networks of tumor cells and how their expression depends on the tumor microenvironment of different tumor models in efforts to find and evaluate key players of tumor suppression.

Funding: HZI Research Group, Indo German Research Project

Zurück an: kruse.susanne@mh-hannover.de
Tumor progression depends on genetic alterations intrinsic to cancer cells and microenvironment of advanced tumors. We recently showed that IRF-1 expression in tumor cells mediate tumor suppressive effects by intrinsic mechanisms leading to stimulation of the immune system. The type of immune response is varies with tumor types. While a solid tumor model causes IRF-1 to induce a protective T cell response, expression of IRF-1 in a pulmonary lung metastasis model is dependent on NK cell dependent elimination of tumor cells by TRAIL and DNAM-1 dependent mechanism. Therefore, IRF-1 represents a signaling pathway that mediates crosstalk between tumor cells and the tumor microenvironment. The aim of the project is to characterize the mechanisms by which IRF-1 influences the microenvironment to drive tumor-specific immune responses.

1. Impact of IRF-1 on liver metastasis.
Preliminary results in the liver metastasis model revealed that IRF-1 expression abolishes liver metastasis by a mechanism distinct from the lung metastasis model. In the initial steps, liver infiltrating immune cells will be identified and characterized. With depletion experiments and use of mutant mice, immune cells which are responsible for the tumor cell elimination will be identified. The identified immune cells will then be reisolated and tested for function and interaction with other immune cells. Finally analysis of tumor cell recognition and effector cell functions in vitro and in vivo will reveal greater insight into the mechanism of tumor elimination.

2. Influence of the tumor microenvironment by IRF-1
Expression of IRF-1 in different tumor models lead to tumor suppression by different mechanisms. We will perform gene expression analysis of IRF-1 expressing tumor cells isolated from different microenvironments to identify tumor specific gene expression. In addition, analysis of cytokine and chemokine milieu of the tumors will show if the influence of IRF-1 on the tumor microenvironment is dependent on of the location and development of the tumors. Thus the impact of IRF-1 in this regulatory network to inhibit tumor development will be investigated.

Time schedule
1. Year 1-2 characterization of IRF-1 effects in liver metastasis
2. Year 2-3 comparison of tumor microenvironments

Group Members:
PhD students: R. Nandakumar, K. Finsterbusch, S. Nair; Bachelor student: Laura Knop;
Technician: M. Grashoff,

Key References for project

Own references (mainly 2009-2011):

Zurück an: kruse.susanne@mh-hannover.de


15.) Name: PD Dr. rer. nat. Florian Kühnel  
Institution: Clinic for Gastroenterology, Hepatology and Endocrinology  
Dept. Leader: Prof. Dr. M. P Manns  
Telephone: +49 (511) 532-3995  
Email: kuehnel.florian@mh-hannover.de  
Name of two Co-supervisors (from different departments): Prof. Dr. A. Heim (Dept. Virology), Prof. Dr. Martin Sauer (Pediatric Hematology/Oncology)  

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: ☐  
Name of student(s): Co-Supervisor of Sumadi Lukman Anwar  
Lecturer: ☐; PhD Kommission: ☐; No, not yet: ☒X, only Co-Supervisor  

Animal experiments involved: ☒ yes  ☐ no  

Research focus:  
The research goal of the group is to develop, and to improve tumor-selectively replicating adenoviruses and to investigate their use as part of oncolytic virotherapy and vaccination approaches for the treatment of solid tumors. During the recent years we have generated several tumor-selectively replicating adenoviruses addressing telomerase activation and/or p53-dysfunction since these alterations are crucial for carcinogenesis. We achieved tumor-specific restriction of viral replication by implementing tumor-specific promoters, protein-mediated repression and antiviral microRNA networks. We could further demonstrate that virotherapy can be combined with systemic chemotherapy to improve therapeutic success. Additionally we are working on the promising use of oncolytic adenovirus therapy as immunotherapeutic approach since oncolytic adenoviruses not only efficiently lyse tumors but can also provoke a substantial antitumoral immune response and can support tumor-directed DC-vaccination. A further aspect of our work is the modification of the viral cell tropism to improve viral infection in tumor tissue. We are not only working on masking the natural liver tropism but also on improving affinity of oncolytic adenoviruses to tumor cells.  

Specific project and methods applied:  
Title: Redirecting virus-specific CD8T-cells to tumors by TCR transfer  
Aims: In the proposed project we want to provoke virus-specific CD8 T cells by viral infection or using virus-peptide pulsed DC-vaccination. Assuming that T cells elicited by a viral infection are provided with favorable cytotoxicity and survival properties, we want to use these cells for retroviral transduction with a TCR receptor that recognizes a tumor-associated model antigen. Thus we want to generate functionally-optimized T-cells for tumor-directed immunotherapy to support the therapeutic outcome of intratumoral virotherapy.  
Funding: DFG, Deutsche Krebshilfe  

The central challenge of oncolytic virotherapy is the survival of a significant proportion of tumor cells until the patients immune system has recognized and eliminated all of the therapeutic vector. Aim of the proposed project is the activation of tumor-specific CD8-T cells by adenovirus-mediated oncolysis in syngeneic murine mouse models of HCC. For this purpose, we want to generate first virus-specific, MHC-I restricted CD8 T-cells by antiviral DC-vaccination and or adenoviral infection. Virus-directed CD8 T-cells are sorted by Pentamer or by DimerX (epitopes for E1A/E1B/dbp) and are transduced in vitro with a heterologous T-cell receptor (OT1-TCR). The OT1-TCR is able to bind the tumor model antigen OVA-derived SIINFEKL peptide bound to MHC class I molecules. Following in vitro
expansion, those chimeric T-cells are then adoptively transferred in non-lethally myeloablated mice bearing OVA-expressing HCC tumors. Adoptive T-cell transfer will be performed in the context of an intratumoral virotherapy. It is our hypothesis that tumor-redired CT8-T cells, provided with superior cytotoxicity/survival/homing properties due to their antiviral shaping, should be well suited to find and kill non-infected tumor cells during a simultaneous intratumoral virotherapy. To allow for a realistic estimation of therapeutic efficacy, we want to investigate this strategy also in naturally grown tumors that have been generated by retroviral/or hydrodynamic transfer of oncogene expressing constructs. Both subcutaneous tumors affected by tumor-selective replication as well as oncogene-induced liver tumors express OVA as tumor-associated model antigen. This strategy investigates the efficacy of immunotherapy by oncolysis and optimally shaped chimeric CD8 T cells in the context of an oncolytic virotherapy.

**Time schedule**
1. First year: generation and sorting of virus-specific T cells, generation of optimized OT1/CD3 expressing retroviruses, establishment of TCR transfer in antiviral T cells, phenotypic and functional characterization, if applicable optimization, establishment of recipient models (choice of reagents for non-lethal myelodepletion in tumor-bearing mice).
2. Second and third year: Adoptive transfer experiments in syngeneic models with subcutaneous Hepa1-6-Ova tumors and Hepa1-6-Ova lung colonies as surrogate for metastasation. Investigations on therapeutic efficacy (survival, tumor size measurements), in vivo characterizations transferred T cells such as cytotoxicity profiles, effector/memory subtypes and ability for homing to virally-infected tumor tissue.

Later (second and third year), this strategy is used to establish a virotherapy-supported, adoptive T-cell Therapy in the oncogene-induced naturally grown in situ model of HCC.

**Group Members:**
Norman Woller (PhD), Engin Gürlevik (PhD), Bettina Mundt (MD), Arnold Kloos (PhD student) Sarah Knocke (technician), Nina Strüver (technician), Paul Schirmer (MD student), Anneliese Goez (MD student), Konrad Ohnhäuser (MD student)

**Key References for project**


**Own references (mainly 2009-2011):**


Zurück an: kruse.susanne@mh-hannover.de
16.) Name: Ulrich Martin  
Institution: LEBAO  
Dept. Leader: Prof. Dr. Ulrich Martin  
Telephone: ++(49) 511-532-8820/8821  
Email martin.ulrich@mh-hannover.de

Name of two Co-supervisors (from different departments):

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: X  Name of student(s): Susan Müller  
Lecturer: ☐;  PhD Kommission: X;  No, not yet: ☐

Animal experiments involved: ☐ yes ☒ no

Research focus:
In the section LEBAO one major research focuses on the differentiation of cardiomyocytes and airway epithelial cells from stem and progenitor cells as well as on the investigation of immunological aspects of regenerative therapies. The basis for the development of novel cell-based therapies for the treatment of cardiac and pulmonary diseases is primarily formed through investigations on a molecular and cellular level of the pluripotent cells. Thereby, our research not only focuses on adult resident stem cells and ES cells, but rather on iPS cells, being an emerging tool for disease modelling, drug screening and patient-specific therapies. ES and iPS cell-derived cardiomyocytes are used for the production of a bioartificial heart muscle whereas other projects aim at the development of stem cell-based biological cardiac pacemaker or cellular treatment of genetic lung diseases. In addition, the establishment of efficient and cell type-specific gene transfer methods, in particular for the application in different stem cell types, are key technologies in this area of operation.

Specific project and methods applied:
Title: TALEN-based targeted transgene integration into safe harbour sites: development of a novel system for generation of multi-transgenic human iPSC lines with pre-defined levels of transgene expression

Aims: Human pluripotent stem cells (hPSCs) are a prime cell source for regenerative therapies due to their extensive expansion potential and the ability to differentiate into essentially all somatic lineages in vitro. The introduction of transgenes into hPSCs will facilitate their pre-clinical testing and other applications such as purification of desired cell lineages during differentiation and in vivo monitoring of transplanted progenies in relevant animal models. To date, generation of clinically applicable transgenic cell lines is limited not only by low transfection rates but particularly by risks of insertional mutagenesis, inefficient site-specific integration of transgenes as well as unpredictable (integration-site-dependent) transgene expression levels and silencing effects.

Very recent progress in zinc finger and TALE nuclease technologies now allows for efficient site-specific integration into the genome of ES and iPS cells. As a straightforward new approach, we have now established highly efficient site-specific transgene integration in human iPSCs.

Aiming at the development of a general TALEN-based site-specific transgene integration approach, it is the goal of this project to construct TALE nuclease specific for various known
safe harbor sites, to establish efficient TALEN-based targeting and to comparatively assess transgene expression levels at these sites. The ability to efficiently introduce transgenes at specific safe genomic loci with well defined expression levels not only represents a straightforward approach for generation of multi-trangenic pluripotent stem cell lines, but will enable the parallel expression of several trangenes with defined expression levels and may even be allow the generation of clinically applicable transgenic cell lines.

**Funding:** institutional resources, application for third party funding underway

**Time schedule**
1. Construction of TALE nucleases for safe harbor sites (months 1-12)
2. General testing of TALE nucleases (months 3-15)
3. Establishment of the TALE nucleases in human iPSCs (months 6-18)
4. Introduction of various transgenes into safe harbor sites of several hiPSC lines and comparative analysis of transgene expression (months 9-36)

**Group Members:**
Stephanie Wunderlich, postdoc
Alexandra Haase, postdoc
Sylvia Merkert, PhD student
Anett Witthuhn, PhD student
Katarzyna Osetek, PhD student
Jennifer Beier, technician
Tim Kohnr, technician

**5 key references for project (including work from other groups)**

**Own references (last 3 years only):**


Research focus:
Aberrant NRas signaling is frequently found in human myeloid leukemia and can be induced by activating NRas mutations as well as by mutations in receptors or signaling molecules upstream of NRas. NRas mutations are most commonly found in acute myeloid leukemias (10-15%, AML), chronic myelomonocytic leukemia (CMML, 20-50%) and juvenile myelomonocytic leukemia (JMML, 20%) (1, 2). However, mutations in cytokine receptors (e.g. FLT3, CSF-1R) or mutations in regulators of Ras signaling such as CBL and NF1 are also often associated with AML, CMML and JMML.

We developed a murine model that faithfully reflects human NRas-induced AML. Using retroviral gene transfer, we identified genes such as the proto-oncogenes Evi1 and its homologue Prdm16, that were activated by insertional mutagenesis and which cooperated with NRas to induce leukemia (3). An independent project in our Institute (O. Kustikova, C. Baum) has established the transcriptional and functional response of primary murine hematopoietic cells to inducible over-expression of Evi1.

EVI1 and PRDM16 expression is frequently activated in human AML by rearrangement of the respective genomic locus. In patients with myelodysplastic syndrome (MDS), a pre-leukemic disease stage, elevated EVI1 expression correlates with increased risk of transformation to AML. In addition, NRas mutations were found to coincide with overexpression of EVI1 and PRDM16 in human leukemia (4, 5). A further cooperation partner for NRas-induced leukemias is AML1/ETO, the fusion protein that results from a translocation t(8;21). Co-expression of both proteins in human CD34 cord blood derived cells lead to leukemia outgrowth in a mouse model (6).

Transformation due to vector-induced insertional mutagenesis is one major drawback of human gene therapy. EVI1 is a frequent target in gene therapy-induced severe adverse reactions, PRDM16 upregulation has been associated with premalignant clonal expansion (7-9), and cooperation with additional mutational events is also a pre-requisite for full transformation in gene therapy associated leukemia. Thus, deciphering the cooperative mechanisms leading to leukemic transformation will also improve our understanding of gene therapy-induced adverse reactions and facilitate their prevention.

Specific project and methods applied:

Title: Oncogene cooperation in myeloid leukemias with hyperactive NRas signaling
**Aims:** The major goal of this project is to study the cooperative mechanisms of Evi1/Prdm16 and other proto-oncogenes such as AML1-ETO in NRas induced leukemia. Expression of Evi1 and Prdm16 are believed to block differentiation and promote cell survival by inducing the expression of antiapoptotic proteins. Both proteins are highly expressed in hematopoietic stem cells. Therefore, Evi1 and Prdm16 may supply the self-renewal capacity needed for long term maintenance of leukemic stem cells. However, during leukemic transformation expression is sustained throughout the myeloid lineage. Furthermore, Evi1 is an epigenetic modulator which interacts with histone methyltransferases and histone deacetylases.

In this project, we will develop models that allow the study of oncogene cooperation. Experiments will be performed in vitro in competitive proliferation assays with primary hematopoietic cells and in vivo in bone marrow transplantation models in syngeneic mice and humanized mouse models (NOD/SCID/Il2Rg-/- (NSG) mice). Furthermore we will address alterations in differentiation, cell cycle progression and cell survival using antibody stainings and FACS flow based methods. Serial bone marrow transplantations will give insights into leukemia progression.

To express the oncogenes we will use retroviral gene transfer. In addition to the established expression system we will develop different retroviral and lentiviral vectors that will direct the expression to different blood lineages (myeloid cells, hematopoietic stem cells, hematopoietic progenitors) by using lineage-specific cellular promoters. Using these vectors will allow more physiological expression levels and will more closely mimic human leukemia.

Since Evi1 was shown to directly interact with the histone methyltransferase Suv39h1 (10), we will also perform experiments in Suv39h1 deficient mice to address the question whether this interaction is essential for the cooperative effect of Evi1. The Suv39h1 mice are already in use in the institute and the breeding established. Furthermore, we will investigate how the signaling profile in leukemic cells may be altered due to additional oncogene expression or different mutations in NRas.

Our final aim, based on the knowledge gained in this project, is to establish in vitro and in vivo mutagenesis models that can be used to explore the impact of gene therapy on induction of myeloid leukemia (similar to our established murine In Vitro Immortalisation (IVIM) Assay (11)). Ideally, these models should be based on human hematopoietic cells.

**Funding:** DFG and others

**Time schedule**
1. Construction and validation of Evi1, Prdm16 and AML1-ETO expressing vectors (first six months)
2. In vitro proliferation, differentiation and clonogenicity assay of Evi1, Prdm16 and AML1-ETO expressing cells in cooperation with increase NRas signalling (first and second year).
3. Bone marrow transplantation of hematopoietic stem cells coexpressing Evi1, Prdm16 or AML1-ETO together with NRas. The latency, phenotype, clonality and progression of these leukemias will be analysed (first and second year).
4. Leukemic cells will be isolated from the mice. To uncover the mechanisms of cooperation we will analyse (1) the contribution of myeloid progenitors and changes in the hematopoietic stem cell compartment, (2) expression profiles in leukemic stem cells by micro array analysis (3) differences NRas downstream signalling by phosphor flow and Western blot analysis. (second and third year).

**Group Members:**
PhD student: Saskia Kohlscheen, Reinhard Hämmerle,
Postdoc: Susanne Wolf, Michael Rothe
Technician: Sabine Knöß, Gabi Paul
About 30 additional members of the Institute of Experimental Hematology

**Key References for project**


* Own references
18.) Name: Prof. Dr. med. Thomas Moritz  
Institution: Rebirth AG Reprogramming  
Dept. Leader: Prof. Dr. med. Thomas Moritz  
Telephone: ++(49) 511-532-5263/64  
Email moritz.thomas@mh-hannover.de

Name of two Co-supervisors (from different departments):

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: x Name of student(s): Pfaff N, Phaltane R, Brennig S  
Lecturer: □; PhD Kommission: □; No, not yet: □

Animal experiments involved: x yes □ no

Research background and focus:
For a number of malignant diseases such as acute myeloid leukemias, breast cancer, or colon cancer a crucial role of tumor stem cells in the origin and the maintenance of the disease has been shown. This project aims to investigate the role of Tumor Stem Cells in Ewing’s sarcoma. Ewing’s sarcoma is the most frequent malignancy of the bone in childhood and is characterised by a specific activating translocation of the Ewing’s sarcoma (Ews) gene, which in > 90 % also involves the Fli-1 gene. Mesenchymal stroma/stem cells (MSCs) have been advocated as the physiologic pendant to Ewing’s sarcoma cells and the modeling of Ewing’s sarcoma including the generation of Ewing’s sarcoma stem cells (ESSCs) has been described following the forced expression of the Ews/Fli-1 transgene in MSCs. A crucial role of the reprogramming factors Sox-2 has been described in this model (Riggi, 2011), and therefore our first aim is to assess the effects of other reprogramming factors such as oct4, nanog, c-myc, or klf4 (Takahashi, 2006) in this context. In addition, we will investigate other approaches for ESSC generation. As recently iPSC generation and maintenance of the original disease phenotype upon differentiation has been described for selected acquired malignancies such as chronic myeloid leukemia or the myeloproliferative syndromes (Carette 2010) we will explore mesenchymal differentiation of either Ews/Fli-1 transduced iPSC/ESC or Ewing’s sarcoma cell derived iPSCs (carrying the Ews/Fli-1 translocation) as alternative ways to generate Ewing’s sarcoma (stem) cells. Our last approach to ESSCs generation will be the isolation of potential stem cells of Ewing’s sarcoma cell lines or primary Ewing’s sarcoma cell cultures using “established” tumor stem cell markers such as CD133, CD44, CD166, or c-kit.

Specific project and methods applied:

Title: Generation and analysis of Ewing’s sarcoma stem cells (ESSC) and the role of reprogramming associated factors in this population.

Aims and work program
Work package (WP) 1: Role of transcription factors in the reprogramming of Ewi/FLi-1 transduced MSCs to ESSCs. Lentiviral vectors expressing the reprogramming associated transcription factors oct4, sox2, nanog, klf4, lin26, or c-myc either alone or in various combinations are available and will be used for the transduction of MSCs in combination with or after transduction with an Ews/ Fli-1 expressing construct. The primary read out for “ESSC-like” cells will be in vitro clonogenic growth and sphere formation (Riggi, 2011)
while confirmatory analysis will employ tumor growth in immunodeficient NSG mice. In addition, to better understand the marked differences in the reprogramming potential between normal (MSC) and tumor (sarcoma) cells we will perform a thorough time course analysis of reprogramming events in Ews/Fli-1 transduced versus normal MSCs including silencing of exogenous reprogramming factors, induction of endogenous reprogramming factors or pluripotency associated marker genes or repression of MSC or associated marker genes. These studies will include gene-specific analysis of promoter methylation using bisulfate sequencing and of histone methylation/acetylation using chromatin-immunoprecipitation (ChiP). In addition, if iPSC-like cells will be generated, these will be further characterized according to standard iPSC criteria.

**WP 2: Mesenchymal differentiation of Ews/Fli-1 transduced iPSC/ESC or Ewing’s sarcoma cell derived iPSCs.** Studies will utilize established in vitro differentiation protocols, which have proven successful for the generation of MSCs from ES/iPS cells (Sanchez, 2011; Giuliani 2011). Again the primary read out for “ESSC-like” cells will be in vitro clonogenic growth and sphere formation and confirmatory analysis will be performed in NSG mice. Naturally, part two, i.e. the mesenchymal differentiation of Ewing’s sarcoma cell derived iPSCs, only will be possible, if these iPSCs are obtained within WP 1.

**WP 3: Identification of ESSCs within Ewing’s sarcoma cell lines or primary Ewing’s sarcoma cell cultures.** For these studies a large panel of Ewing’s sarcoma cell lines as well as established Ewing’s sarcoma primary bulk cultures (U. Dirkssen, Münster; German Ewing’s sarcoma study group) is available. Preliminary data indicate that several of these lines/cultures indeed contain a small portion of primitive CD133-positive “ESSC-like” cells and again both, in vitro clonogenic growth analysis for sphere formation and in vivo studies in NSG mice will be performed on these populations.

**Funding: REBIRTH Funds**

**Group Members:** (either indicate the name(s) or the number of staff)
- 2 postdoc;
- 3 PhD student;
- 2 Diploma or Master student;
- 1 technician

**Key References for project**


**Own references (mainly 2009-2011):**

Zurück an: kruse.susanne@mh-hannover.de


Zurück an: kruse.susanne@mh-hannover.de
19.) Name: PD Dr. Margarete Niebuhr
Institution: Dept. of Dermatology and Allergy, Division of Immunodermatology and Allergy Research
Dept. Leader: Prof. Dr. Thomas Werfel
Telephone: ++(49) 511-532-5092
Email niebuhr.margarete@mh-hannover.de, werfel.thomas@mh-hannover.de

Name of two Co-supervisors (from different departments):
   1) Prof. Dr. Wolfgang Bäumer, Department of Pharmacology, University of Veterinary Medicine in Hannover
   2) Prof. Dr. Armin Braun, Department of Immunology, Hannover Medical School

Engagement in the PhD program Molecular Medicine or HBRS:
Co-Supervisor: X Name of student(s): Sadaf Kasraie
Lecturer: □; PhD Kommission: □; No, not yet: X

Animal experiments involved: □ yes X no

Research focus:
Our research group focuses on the impact of Staphylococcus aureus (S. aureus) in the pathogenesis and maintenance of atopic dermatitis (AD) and psoriasis which are very common chronic inflammatory skin diseases affecting around 2-5% of adults. A hallmark of AD is the striking susceptibility to S. aureus colonization of the skin. A positive correlation exists between disease severity and S. aureus colonization of both lesional and nonlesional skin. Moreover, many AD patients suffer from bacterial superinfections with this germ with a number of frequently observable clinical complications. Therefore, our research group wants to elucidate differences in elements and mechanisms of the innate and adaptive immune system in AD as compared to healthy conditions in respect to staphylococcal components to contribute new knowledge on the pathogenesis and maintenance of this disease.

Specific project and methods applied:
Title: Inflammasome associated signalling in myeloid cells from patients with chronic inflammatory skin diseases

Scientific objective: Investigation of inflammasome dependent mechanisms which may be altered in patients with resistant AD, may contribute to the chronification of the disease and to the susceptibility of patients with AD to cutaneous microbial colonization and infections.

Aims: The major aim is to better understand the complex interplay between stimuli, substrates and elements of the inflammasome in human myeloid cells and to define their plasticity in that respect in the milieu of inflamed skin. Findings with cells or tissue from AD patients will be compared to psoriasis and healthy conditions to elucidate clinically relevant defects in innate immunological mechanism in AD.

Methods: (i) Isolation and cell culture (macrophages, inflammatory dendritic epidermal cells [IDEC], monocyte derived myeloid dendritic cells, Langerhans cells, T cell lines and clones from blood and skin and primary keratinocytes cultured from epidermal stem cells of the hair follicle or from skin biopsies keratinocytes, T-cells). (ii) Investigation of gene expression (quantitative mRNA PCR, Pyrosequencing). (iii) Protein expression will be investigated by ELISA, Flow cytometry and Western Blot. The binding activity of transcription factors will

Zurück an: kruse.susanne@mh-hannover.de
be determined by EMSA. (iv) Manipulation of gene expression: siRNAs and/or shRNA containing plasmids targeting NLRP3, ASC and Caspase-1 mRNAs will be used for silencing of the adequate target genes. (v) Laser microdissection to distinguish cells regarding their (in vivo) inflammasome dependent inflammation status.(vi) 2D, 3D skin models and ex vivo skin culture: Cocultures with myeloid cells, T cells and keratinocytes will be performed to translate major findings into 2D and 3D models of dermatitis.

Background / Abstract
Atopic dermatitis (AD) and psoriasis are very common chronic inflammatory skin diseases affecting around 2-5% of adults. A hallmark of AD is the striking susceptibility to Staphylococcus aureus (S. aureus) colonization of the skin. A positive correlation exists between disease severity and S. aureus colonization of both lesional and nonlesional skin. Although some patients with psoriasis are colonized with S. aureus as well only AD patients suffer from bacterial superinfections with a number of frequently observable clinical complications. This implicates differences in elements and mechanism related to the innate immune system in AD as compared to psoriasis and healthy conditions.

Due to our preliminary findings we propose to focus on inflammasome molecules (mainly with regard to the NLRP3 inflammasome) and inflammasome-dependent inflammatory cytokines which may be altered in human myeloid cells in AD. Besides defining the expression of those molecules in vitro and in inflamed skin, we plan to establish genetic and epigenetic differences in the inflammasome complex in cutaneous myeloid cells that may explain local deficiencies in combating S. aureus in AD. The plasticity of myeloid cells shall be investigated by comparing key findings of inflammasome expression and function in conjunction with cells contributing to dermatitis, i.e. activated keratinocytes and skin infiltrating T-lymphocytes. Here we aim to perform cocultures in 2D and 3D model systems and ex vivo skin cultures from normal and lesional skin representing a more physiological scenario of cutaneous inflammation than in vitro cultures.

The project is of high clinical relevance because up to now no efficient long term therapeutical approach is available for severely affected patients with persistent AD who are known to suffer frequently from bacterial skin infections with S. aureus which in turn aggravates dermatitis. Thus, the elucidation of a local immune dysregulation due to plasticity of myeloid cells or to inherited defects in the inflammasome complex may lead to new concepts and identify new therapeutic targets for severe AD and related skin diseases.

Funding: DFG grant proposal is under review, industrial funding

Work program / Time schedule
1. Characterization of the expression of inflammasome molecules in myeloid cells in vitro and in situ.
2. Investigation of genetic and epigenetic differences in molecules of the inflammasome responsible for reduced innate immune reactions in AD.
3. Elucidation of the responsiveness of myeloid cells to stimuli and of substrates of the inflammasome.
4. Determination of key functions of the inflammasome of cutaneous myeloid cells.
5. Defining the plasticity of myeloid cells in 2D and 3D models and in ex vivo cultures of lesional skin.
Group Members:
Kathrin Baumert, technician

Key References for project


Own references (mainly 2009-2011):

Zurück an: kruse.susanne@mh-hannover.de
Name: Prof. Dr. R. Schwinzer
Institution: Transplant Laboratory, Dept. of General-, Visceral- and Transplant. Surgery
Dept. Leader: Prof. Dr. J. Klempnauer
Telephone: +(49)511-532-4204
Email schwinzer.reinhard@mh-hannover.de

Name of two Co-supervisors (from different departments):
Prof. Dr. H. Niemann, Abtlg. Biotechnologie, Mariensee
Prof. Dr. T. Witte, Abtlg. Klinische Immunologie, MHH

Engagement in the PhD program Molecular Medicine or HBRS:
Supervisor: ☐ Name of student(s):
Lecturer: X; PhD Kommission: ☐; No, not yet: ☐

Animal experiments involved: X yes ☐ no

Research focus:
The research of our group focuses on transplantation biology. We characterize immune responses to allo- and xenografts on cellular and molecular level to identify new targets for therapeutic immunodulation and/or tolerance induction. In vitro assays using cells from patients after clinical kidney and liver transplantation was well as genetically defined rat models of allo- and xenotransplantation are applied. Recent data from our group and others indicate that enhancement of negative costimulatory signals could be an approach to downregulate immune responses to allo- and xenografts. The significance of this concept is currently evaluated.

Specific project and methods applied:

Title:
Negative costimulation for the control of cellular immune responses to porcine xenografts

Aims:
We have previously shown that porcine cells genetically engineered to overexpress the negative costimulator PD-L1 induce diminished T cell responses in vitro and also in vivo as observed in a pig-to-rat cell transplantation model (pig cells under rat kidney capsule). It is the aim of the project to test the hypothesis that xenografts delivering strong negative costimulatory signals to recipient T cells require only moderate immunomodulation for long-term survival. Following this concept we will study the efficiency of pharmacological immunosuppression and antibody-mediated costimulatory blockade to prolong survival of PD-L expressing cells and controls. Furthermore, to make the PD-1/PD-L approach accessible for pig-to-primate transplantation independent of the availability of PD-L transgenic pigs we will generate soluble agonistic PD-Ligands and assess their biologic activity. To address these aspects, the following specific aims/questions will be pursued:

1. Characterization of the mechanisms leading to diminished immune responses of rats to PD-L expressing cellular grafts. Based on data from in vitro experiments we hypothesize
that CD4⁺CD25⁺ regulatory T cells are involved. Graft infiltrating cells will be analyzed by immunohistochemistry and flow cytometry. In vitro tests will be performed to study their function.

2. Can long-term survival of porcine grafts exhibiting a strong negative costimulatory potential be achieved by minimal immunomodulation? The efficiency of calcineurin and mTOR inhibitors as well as costimulatory blockade by pig-specific mAb to prolong survival of PD-L expressing cells and controls will be assessed.

3. Enhancement of negative costimulation by soluble PD-Ligands. Expression vectors will be constructed containing the extracellular domain of human or rat PD-L1 or PD-L2 combined with the Fc portion of human immunoglobulin. The inhibitory potential of PD-L.Ig molecules will be assessed by analyzing their effects on human anti-pig and rat anti-pig in vitro responses (proliferation, cytokine secretion etc.) and rat anti-pig in vivo reactivity.

Funding:
DFG

Time schedule:

2. Effect of additional immunomodulation (CsA, blocking anti-pig mAb) on survival of PD-L grafts.
3. Generation of expression vectors and transfectants producing PD-L.Ig, purification of recombinant proteins; assessment of the biologic activity of negative costimulators by in vitro test systems.
4. Analysis of the therapeutic potential of PD-L.Ig in pig-to-rat model.

Group Members:

Wiebke Baars, Technician; Katja Borns, Technician; Sonja Kolllrich, Technician; Dr. Claudia Pokoyski, Postdoc; Dorothee Römermann, Biologist; Dr. Sascha Rother, Postdoc; Maria Wicker, PhD-student

Key References for project


Zurück an: kruse.susanne@mh-hannover.de

**Own references (mainly 2009-2012):**


* joint authorship.


21.) Name: Julia Skokowa  
Institution: Dept. of Molecular Hematopoiesis  
Dept. Leader: Prof. Dr. med. Karl Welte  
Telephone: ++(49) 511-532-9210  
Email Skokowa.julia@mh-hannover.de  
Name of two Co-supervisors (from different departments): Axel Schambach, Matthias Eder

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: ☐ Name of student(s): Olga Klimenkova  
Lecturer: ☐;

Animal experiments involved: ☐ no

Research focus:  
Severe congenital neutropenia (CN) is a heterogeneous disorder of hematopoiesis characterized by a maturation arrest of granulopoiesis in bone marrow at the stage of promyelocytes. There are two major subtypes of CN as judged by inheritance type: autosomal dominant trait defined by mutations in the elastase 2 (ELANE) gene, encoding the neutrophil elastase (NE) protein consisting of 60% of patients and autosomal recessive trait comprising approximately 30% of patients. In some cases, the same ELANE mutations have been observed in the 2 different syndromes, CN and cyclic neutropenia (CyN). CyN patients usually have regular oscillation of blood neutrophil counts with periods of severe neutropenia occurring every 21 days. Intriguingly, other blood cells (monocytes, erythroblasts, megakaryocytes) also have regular cycles, suggesting presence of abnormal hematopoietic signalling cascades already in early hematopoietic stem cells in CyN patients. In contrast, CN patients have isolated defects in granulocytic progenitors only with unaffected numbers and morphology of cells of other hematopoietic lineages. Both CN and CyN could be successfully treated with G-CSF. However, G-CSF treatment does not eliminates 21-day oscillations of granulocyte numbers. Moreover, CN is a pre-leukemic syndrome with cumulative incidence to develop leukemia of app. 20 %, but CyN patients do not develop leukemia. It is unclear, how mutations in the same amino acid of the same gene (ELANE) induce two completely different diseases. In our European Branch of the Severe Chronic Neutropenia International Registry (SCNIR), which is situated in our department, data and tissue samples are available of 336 CN and CyN patients. This gives us a unique opportunity for a comprehensive multifactorial analysis of the pathomechanisms leading to CN or CyN.

Specific project and methods applied:  
Title: Evaluation of the “co-mutated” gene/s responsible for the phenotypic differences between patients with congenital and cyclic neutropenia  
Aims: We aimed to identify the co-mutator gene/s that in combination with ELANE mutation cause either CN or CyN phenotype.  
We already identified families of CN and CyN patients carrying same ELANE mutations. We collected DNA and partially performed Next Generation Sequencing (NGS) of the DNA samples from these families. More families have to be sequenced. We used SOLID and Complete Genomics (CG) sequencing platforms.  
We will analyse data and validate mutated candidate genes by Sanger sequencing. Candidate genes will be filtered using different filtering approaches already established in our laboratory.  
Functional consequences of the mutations in the candidate genes will be further analysed:
1. We will evaluate the effects of the mutations in candidate genes on their specific downstream target genes/signaling cascades. For this we will analyze mRNA expression data obtained from microarrays of RNA samples from families used for NGS (microarray data for some families are already available) with followed validation by qRT-PCR and functional studies.

2. We will make WT and mutated cDNA of each candidate gene and study the effects of each gene mutations on the in vitro hematopoietic differentiation of primary human hematopoietic cells of healthy individuals using CFU assays and liquid culture.

3. We will evaluate the effects of candidate gene mutations in rescue experiments of hematopoietic cells of CN and CyN patients with WT cDNA of candidate genes. We will also use iPS cells of CN and CyN patients generated in another project.

**Time schedule**

1. Identification of mutated genes, validation by Sanger sequencing (app. 6 months).
2. Evaluation of the effects of the mutations in candidate genes on the specific downstream signaling pathways, functional studies (app. 1 year).
3. Evaluation of the effects of mutated candidate genes on the in vitro hematopoietic differentiation (app. 6 months - 1 year).

**Group Members:**

PhD students: Olga Klimenkova, Maxim Klimenkov, Bardia Samareh, Vaso Basinou; Research fellow: Inna Kusnetsova; Bioinformatics: Sergej Kandabarov; Diploma or Master student: Gleb Karachunksky; Medical student: Yannick Bruns; Technicians: Annette Müller Brechlin, Marlene Reuter, Anna Giginia, Anna-Lena Hagemann, Anna Klaus.

**Key References for project**


**Own references (mainly 2009-2011):**

22.) Name: Beate Sodeik  
Institution: Institute of Virology, Hannover Medical School  
Dept. Leader: Prof. Dr. Thomas F. Schulz  
Telephone: ++(49) 511-532-  
Email  
Name of two Co-supervisors (from different departments):

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: Name of student(s):  
Lecturer: X; PhD Kommission: X (in the past); No, not yet: ☐

Animal experiments involved: ☐ yes X no

Research focus:  
Herpes Simplex Virus (HSV) infection of epithelial, dendritic and neuronal cells; Genetics, biochemistry and cell biology of the required virus-host interactions  
Neurobiology: Role of axonal transport for the pathogenesis of HSV; Immunology: PAMPS of HSV1 – Cytosolic host restriction factors; Cell biology: Digital time lapse microscopy of wild-type and mutated HSV1 strains tagged with fluorescent proteins on the capsid (VP269) and the tegument (pUL37, VP11/12, US11) or the envelope (gD); immuno-electron and light microscopy.  
Function of the actin and microtubule cytoskeleton and molecular motors during herpes simplex virus entry, assembly and egress; Nuclear targeting of the herpes simplex virus genome to the nuclear pore complex;  
Biochemistry: Cell-free in vitro assays for microtubule mediated viral capsid transport and nuclear pore targeting and viral genome uncoating; Molecular biology: Bacterial artificial chromosomes for the targeted mutagenesis of Herpes Simplex Virus type 1; GST fusion proteins, transient transfection

Specific project and methods applied:  
Title: Cytosolic host restriction factors recognizing incoming HSV1  
Funding: DFG-SFB 900, LOM funds

Background  
Alphaherpesviruses such as Herpes Simplex Virus (HSV) replicate in epithelial cells and keratinocytes of mucosal membranes and skin and establish persistent infections in the sensory nerve ganglia innervating these regions. HSV1 is an enveloped virus with an icosahedral capsid containing the double-stranded DNA genome of 152 kb, and about 30 tegument and capsid-associated proteins located between the capsid and the viral membrane. We study the cell biology of HSV1 in epithelial cell lines, dendritic cells and primary neurons. HSV1 enters cells either by direct fusion at the plasma membrane or by an ill-characterized endocytic mechanism. We have shown that the incoming capsids utilize inner tegument proteins to recruit the microtubule motors dynein, kinesin-I and the cofactor dynactin for active transport to the nucleus. According to the textbook view on HSV1 nuclear targeting, the viral DNA genome is protected during its entire cytoplasmic and cytosolic passage by its capsid until this docks at the nuclear pore, where the viral genome is released into the nucleus for viral transcription and replication.  

However, recent research on innate immunity mechanisms, particularly against herpesviruses, suggest that nucleic acids of HSV1 are sensed by the endosomal Toll-like receptors TLR3 (dsRNA) in epithelial and fibroblastic cell types but not in plasmacytoid

Zurück an: kruse.susanne@mh-hannover.de
dendritic cells, but by TLR9 (DNA) and possibly TLR 7 (ssRNA) in dendritic cells and macrophages. On the plasma membrane of epithelial cells, TLR2 and TLR4 may also contribute to innate immunity by recognizing unknown HSV1 glycoproteins. In the cytosol, there are additional pattern recognition receptors (PRRs) such as RIG-I and MDA5 that detect dsRNA and ssRNA and are activated after the initiation of HSV1 gene expression. Furthermore, several cytosolic and nuclear DNA sensors have been suggested to detect cytosolic DNA during infection with herpesviruses: DAI, RNA polymerase III, AIM2, DHX9 and DHX36, and most recently IFI16. However, in most experiments, the DNA has been transfected directly into the cytosol, and in those cases in which infection experiments have been performed, the biochemical composition of the inoculum has not been well characterized.

As Paludan et al. (2011) wrote “it is not known how the genomic material arrives at these cellular compartments nor how the capsid-protected DNA is made accessible to PRRs”. Endosomal lipases and proteases may disintegrate incoming particles, but the released genomes would still have to be translocated across endosomal membranes; cytosolic proteases may nibble on incoming cytosolic capsids, or viral DNA may leak or be actively exported out of the nucleus after HSV-1 replication. Furthermore, unprotected DNA and other PAMPs may be taken up from neighboring infected cells that have already succumbed to the HSV1 induced cytopathic effects. The cell may also classify the capsids as large protein aggregates that can no longer be refolded, but should be degraded by the proteasome, or if impossible, be sequestered by autophagy for lysosomal degradation.

**AIMS:**
- Characterize the relative contribution of HSV1 DNA, HSV- capsids, and HSV1 glycoproteins to the induction of IFN-β expression in human epithelial cells.
- Identify cytosolic host factors sensing HSV1 PAMPs and characterize their functional relevance.

We will challenge epithelial cells and dendritic cells with HSV1 inoculum of equal MOI but different biochemical composition, and measure the induction of IFN-β. For these studies we will use crude postnuclear supernatants of HSV1 infected cells, as in many immunological studies, or gradient purified virions tagged with fluorescent protein domains, that will either be left untreated or spiked with additional viral DNA, with membrane vesicles from control or infected cells, or with HSV1 capsids. In further experiments using RNAi, we will silence specific PRRs and/or general downstream members of the signaling cascades inducing IFN production. These studies will allow us to evaluate the relative contribution of the recognized and potentially new HSV1 PAMPS for the induction of type I interferon in epithelial cells.

In ongoing work with Lucas Pelkmans (ETH Zürich), we have developed a high-throughput and high-content automated fluorescence microscopy screen to measure gene expression of HSV-1 as well as 16 other viruses in two different HeLa cell lines using GFP as a common transgene (Snijder et al., accepted). Based on this system, we furthermore have screened a druggable library targeting 7,000 host genes, each with 3 individual siRNAs (QIAGEN), and identified 200 candidate host genes that stimulated and about 100 genes that upon silencing reduced HSV1 mediated gene expression. These genes are currently validated in a secondary screen using 3 other siRNAs from a different vendor (AMBION). While we still await further validation, we hypothesize that some of the 200 up-hits contribute to intrinsic host resistance or innate immunity, e.g. subunits of the proteasome, several endosomal and cytosolic proteases, or ill-characterized DNA binding proteins. These host factors may not just “sense” the PAMPs and “signal” to downstream factors, but they may also sequester or inactivate incoming HSV1 particles. According to this hypothesis, their
inactivation by gene silencing should enable more genomes to reach the nucleus and foster HSV1 gene expression. Furthermore, based on the suggestions of Veit Hornung (University of Bonn), we included host genes implicated in intrinsic resistance mechanisms, innate immunity as well as further potential DNA sensors. For this PhD thesis, we aim to identify the molecular functions of the highest ranked 5 to 10 genes of the up-hit group. These experiments shall guide us to the most relevant innate immunity-related proteins of the host cell that contribute to efficient HSV1 infection as well as gene transfer mediated by HSV1 vectors.

**Group Members:**
Fenja Anderson, postdoctoral scientist; Randi Diestel, postdoctoral scientist, Katharina Goris, postdoctoral scientist; Thalea Koithan, PhD student; Anna Buch, PhD student; Deepika Devadas, PhD student; Dagmara Bialy, PhD student Lyudmila Ivanova, PhD student; Ute Prank, technician; Anja Pohlmann, technician part-time; Anne Binz, technician part-time

**Key reviews:**


**Own references (2008-2011):**


23.) Name: Renata Stripecke  
Institution: Dept. of Hematology, Hemostasis, Oncology and Stem Cell Transplantation  
Dept. Leader: Prof. Dr. Arnold Ganser  
Telephone: ++(49) 511-532-6999  
Email stripecke.renata@mh-hannover.de  
Name of two Co-supervisors (from different departments): Prof. Dr. Ralf Gutzmer (Dermatology); Prof. Dr. Tim Sparwasser (Twincore)  

Engagement in the PhD program Molecular Medicine or HBRS:  
Supervisor: X Name of student(s): Mudita Pincha (graduated 2011); Shikhar Aggarwal (since 2011)  
Lecturer: X PhD Kommission: X (India); No, not yet: □  

Animal experiments involved: X yes □ no  
Research focus:  
The Lymphatic Cell Therapy Laboratory develops new translational concepts for immune and lymphatic regeneration. Our target disease is cancer (melanoma and leukemia), and we have established mouse models of cancer development and for hematopoietic stem cell transplantation. Our focus is on dendritic cells (DCs), which are the key entities of the immune system that orchestrate innate and adaptive immune responses and promote a balance between immune activation versus tolerance. Due to cancer, infections and immunosuppressive regimens, the function of DCs is suppressed leading to a failed immune surveillance. Thus, DC immunotherapy is intensively explored to restore the homeostatic and antigenic responses ¹.  

Animal experiments involved: X yes □ no  

Specific project and methods applied:  
Title: Combination of personalized targeted therapy and immunotherapy against melanoma: preclinical mouse model  

Zurück an: kruse.susanne@mh-hannover.de
This project is designed in light of current developments in the standard of care (SOC) for metastatic melanoma. For about half of the patients, the life expectancy with standard chemotherapy treatment remains low – in average 8 months after diagnosis. For the second half, carrying a B-RAF V600 mutation in the melanoma cells, the cancer is highly susceptible to treatment with Vemurafenib (also known as PLX4032 or RG7204) dramatically reducing tumor burden, yet relapse occurs in the majority of patients after a median of around 6 months. Recent work by other groups has demonstrated that: 1. Treatment of melanoma cell lines with B-RAF V600 inhibitor increases the presentation of melanoma differentiation antigens, including TRP2, possibly due to apoptose induction; 2. T lymphocytes infiltrate tumors early following treatment of melanoma patients with B-RAF inhibitor. These findings underscore the potential of synergistic effects of SmartDC-TRP2 targeting potent lymphocyte responses against a relevant melanoma anti-apoptotic gene product (TRP2) to eliminate minimal residual disease and drug resistance variants in melanoma after or during treatment with Vemurafenib. Thus, the hypothesis to be tested in the SIM transplantable mouse melanoma model is whether administration of SmartDC-TRP2 after a period of Vemurafenib treatment can induce potent immune responses to ultimately cure melanoma in mice (Fig. 2).

**AIM:** Establish the SIM mouse model of B-RAF V600 melanoma in C57BL/6 mice and evaluate the effects of Vemurafenib in combination with SmartDC-TRP2 therapy.

**Work plan:** The SIM melanoma cell line (derived from transgenic B-RAF V600 mice developing melanocytic hyperplasia) was previously tested by Prof. Ribas and colleagues (University of California, Los Angeles) after subcutaneous implantation in C57BL/6 mice, showing that palpable tumors on mice could be detected approximately 10-15 days after implantation (Ribas et al, in revision). This cell line has been genetically modified to express firefly luciferase, enabling non-invasive tumor engraftment and progression analyses with optical imaging analyses. Using this model, Ribas et al demonstrated that Vemurafenib (commercially available from Selleck) administration significantly delayed tumor growth and increased IFN-gamma production in the tumor infiltrating lymphocytes. Importantly, although tumor growth was delayed, the mice could not be fully cured with Vemurafenib, which recapitulates the clinical problem. We plan, in order of stringency, the following experiments: 1. Establish the SIM/Vemurafenib model at the MHH; 2. Test the protective effects of SmartDC-TRP2 administration prior to SIM challenge; 3. Ultimately evaluate the effects of Vemurafenib and/or SmartDC-TRP2 therapy (Fig. 3). Vemurafenib and SmartDC-TRP2 will be administered after tumors become palpable (recapitulating clinical treatment of melanoma). Potency will be evaluated by survival to tumor challenge and by anti-TRP2 immune responses (CTL, Th, antibody responses) at different time-points (4, 16 and 26 weeks after tumor challenge). Identity,
potency, immuno-histological analyses of toxicities or tumorigenicities will be evaluated.

**Funding:** Rebirth, Deutsche Krebshilfe.

**Time schedule**
1\textsuperscript{st} year: Establish SIM model of melanoma and treatment with Vemurafenib.
2\textsuperscript{nd} year: Evaluate protective and therapeutic schedules of combined SmartDC-TRP2 and Vemurafenib therapy.
3\textsuperscript{rd} year: Evaluate mechanism(s) of immune responses and biosafety of combined therapy.

**Group Members:**

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<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Country</th>
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<tbody>
<tr>
<td>Renata Stripecke</td>
<td>principal investigator</td>
<td>Brazil, USA, Germany</td>
</tr>
<tr>
<td>Gustavo Salguero</td>
<td>post-doc</td>
<td>Colombia</td>
</tr>
<tr>
<td>Bala Sai Sundarasetty</td>
<td>post-doc</td>
<td>(India)</td>
</tr>
<tr>
<td>Anusara Daenthalinanmak</td>
<td>PhD student</td>
<td>ZIB (Thailand)</td>
</tr>
<tr>
<td>Shikhar Aggarwal</td>
<td>PhD student</td>
<td>Mol Med (India)</td>
</tr>
<tr>
<td>Laura Macke</td>
<td>lab manager/technician</td>
<td>(India)</td>
</tr>
<tr>
<td>Andreas Schneider</td>
<td>lab manager/technician</td>
<td>(Ukraine, Germany)</td>
</tr>
<tr>
<td>Helmut Schwarzer</td>
<td>lab assistant</td>
<td>(Germany)</td>
</tr>
</tbody>
</table>

**Key References for project**
Name: Yulia Kiyan, PhD/ Inna Dumler, Prof., PhD
Institution: Dept. of Nephrology
Dept. Leader: Prof. Dr. med. Hermann Haller
Telephone: ++(49) 511-532-2395; ++(49) 511-532-2715
Email dumler.inna@mh-hannover.de
kiian.ioulia@mh-hannover.de

Engagement in the MD/PhD program or HBRS:
Supervisor: Name of student(s): Krishna Vallabhaneni; Parnian Kalbasi
Lecturer: ; PhD Kommission: ; No, not yet:
Animal experiments involved: yes  no

Research focus:
Our research interest is focused on molecular mechanisms of vascular diseases, such as arteriosclerosis, restenosis, grafts arteriosclerosis, chronic transplant rejections, and aims at novel therapeutic strategies. In particular, we are interested in the multifunctional urokinase (uPA)/urokinase receptor (uPAR) system, which is a critical participant in these processes 1. Our recent studies suggest a new role for the uPA/uPAR system in functional behavior of vascular cells and their interplay upon vascular remodeling following injury through the induction of exclusive signaling cascades and regulation of gene expression 2-6. The research group has an internationally acknowledged expertise in this field. Over the years a large repertoire of experimental tools has been generated including mutant forms and silencing constructs for molecules of interest cloned in different viral vectors that are functional in primary vascular cells.

Increasing body of evidence indicates that the uPA/uPAR system is an active participant in the majority of infection and inflammatory diseases. uPAR expression is increased in cytokine or bacteria activated cells including macrophages and monocytes, and contributes to the infiltration of inflammatory cells into infected tissues 7. uPAR regulates inflammatory responses serving as a modulator of immunocompetent receptors on resident cells and of lymphocyte recruitment to the site of injury 8-12. Recent studies documented that uPAR has direct involvement in the response of inflammatory cells, such as neutrophils and macrophages, to TLR2 stimulation in vitro and in vivo 13 and in efferocytosis, a process critical for the resolution of inflammation and production of anti-inflammatory cytokines 14. Our recent findings revealed one novel unexpected aspect of uPAR in vascular remodeling and inflammation. We found that that modified lipids (such as low concentrations of oxLDL) initiate in an oxidative stress independent fashion atherogenic signals triggering vascular smooth muscle cells (VSMC) transition to pathophysiological phenotype and expression of pro-inflammatory mediators. uPAR was required to mediate these effects, because uPAR deficiency lead to their abrogation. Interestingly, we observed uPAR association with the scavenger protein CD36 in response to oxLDL in VSMC. Though several interacting proteins have been identified for uPAR, which is a GPI-anchored cell surface receptor, its association with CD36 has not been documented so far. These and further findings suggest that a functional complex of uPAR, CD36 and most likely TLR may exist. In VSMC, formation and activation of this functional unit in response to modified lipids may be a mechanism to trigger and propagate the atherogenic inflammation. Identification of this molecular machinery and evaluation of its functional consequences in vivo represent the major goal of the present proposal. The expected results may provide additional new insights into the link among lipids, inflammation and atherosclerosis and important and novel information on the molecular mechanisms underlying the dysregulated atherogenic inflammatory process and failure of effective resolution in the vessel wall. We hope that they may further provide potential therapeutic or preventive targets for cardiovascular diseases.
Specific project and methods applied:
Title: Smooth muscle cells in vascular inflammation: role for the urokinase receptor and modified lipids
Aims:
Ultimate aim of this project is to elucidate molecular mechanisms underlying uPAR-directed pro-inflammatory reactions in vascular cells induced by modified lipids independently of oxidative stress induction. Based on our preliminary research, we hypothesize that VSMC stimulation with modified lipids induces an alternative signaling leading to crucial changes in cell functional behavior and to expression of pro-inflammatory mediators. We further suggest that the underlying molecular mechanism involves formation and activation of the uPAR-directed signaling complex consisting of uPAR, CD36, and TLR, presumably TLR-4 or TLR-2.
Our research addresses the following questions:
- What kind of modified lipids affect VSMC functional and inflammatory properties?
- What are the underlying molecular mechanisms?
- How are these processes regulated?
- What are the functional consequences of these molecular events in vivo?
General experimental approach:
Cellular models
We will use VSMC from human umbilical cord artery freshly isolated and cultured according to laboratory protocols. Additionally, primary human coronary artery VSMC (PromoCell, Germany) will be used. Cell stimulation with oxLDL, minimally modified LDL (mmLDL), and oxidized phospholipids (oxPL) will be performed according to our protocols. Oxidative stress will be controlled by 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence (Invitrogen). A range of lipid concentrations inducing low (negligible) and high oxidative stress will be used for cell stimulation.
Cell differentiation will be monitored by expression of VSMC specific markers, using immunoblotting, immunocytochemistry, FACS- and TaqMan analysis. Transcriptional activity of related genes will be examined in luciferase assays. Cell proliferation will be monitored by the measurements of DNA synthesis BrdU labeling kit III. Migration properties including cytoskeleton rearrangements will be analyzed using Boyden chamber and timelapse imaging. Cell apoptosis will be assessed by cell counting, cell staining with the Hoechst 33258 stain, TUNEL technique, and usage of specific pro- and anti-apoptotic markers as described. Expression of pro-inflammatory mediators will be measured by Quantikine® Sandwich ELISAs.
To define the requirement of uPAR for cell functional behavior and pro-inflammatory properties, the RNA silencing technology for specific uPAR expression inhibition using lentiviral RNA interference vectors and nucleofection technology will be used as worked out in the laboratory and described. The same approach will be used to downregulate other proteins of interest, such as CD36, TLR etc. Further we will use specific inhibiting peptides, receptors antagonists, and neutralizing antibodies. In some experiments VSMC isolated from the aorta of uPAR-deficient and CD36-deficient mice will be additionally used.
Task 1. Effects of modified lipids on cell functional and inflammatory properties
Our preliminary data indicate that low concentrations of oxLDL induce in uPAR-related and oxidative stress independent fashion VSMC phenotypic modulation and expression of pro-inflammatory mediators. Several experimental approaches will be used to expand these observations.
There is evidence that inflammatory response in the vascular wall is initially triggered by so-called “minimally modified/oxidized” LDL (mmLDL), which then is modified into “highly oxidized” LDL. Further studies imply important role for oxidized phospholipids,
which are components of the LDL particles, in induction of vascular inflammation \(^{18}\) and VSMC functions \(^{19}\). Therefore, we will analyze effects of all these components on VSMC functional fate and inflammatory responses. LDL will be prepared from human plasma by ultracentrifugal separation. To produce mmLDL, LDL oxidation by human 15-lipoxygenase will be performed by incubating LDL with 15-lipoxygenase expressing cells as described \(^{18}\). Extensively oxidized LDL will be generated by Cu\(^{2+}\)- induced oxidation according to our standard protocol. It was shown that there are significant similarities between Cu\(^{2+}\)- oxidized LDL and oxLDL found in atherosclerotic lesions \(^{20}\). However, it is still controversial whether Cu\(^{2+}\)-oxidation occurs \textit{in vivo}. Therefore, we will additionally use macrophage-mediated oxidation of LDL as established \(^{20}\). Oxidized specific phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (PAPC), will be obtained by air oxidation of dry PAPC (Sigma-Aldrich) as described \(^{18}\). Cell functional studies will performed as indicated under Cellular Models section.

**Task 2. Structure-functional studies on the proposed uPAR interactome**

\textbf{a) Pilot experiments on the composition of the presumable signaling complex}

Based on our preliminary results and literature data we hypothesize that modified lipids may trigger in VSMC formation and activation of the uPAR-directed interactome consisting of uPAR, CD36, and TLR, to affect VSMC phenotypic transition to proinflammatory phenotype and expression of pro-inflammatory mediators. To verify this working hypothesis experimentally, several experimental approaches will be used. Because we confirmed already uPAR-CD36 association in response to oxLDL, we will next examine whether TLR is a constituent of this signaling complex. Because TLR4 and TLR2 have been identified in VSMC, and TLR2 is able to recognize the most diverse set of ligands including lipoproteins \(^{21}\), we will first determine expression of these TLR in cells stimulated with modified lipids using immunoblotting, TaqMan analysis and immunocytochemistry. Changes in expression of uPAR and CD36 in response to modified lipids will be also examined. Next, we will downregulate uPAR, CD36, and TLR2 or/and TLR4 (depending on the results) using siRNA, blocking peptides and antibodies. As a readout of these experiments, functional assays addressing cell fate and expression of proinflammatory mediators will be used as described in above. If involvement of TLR could not be confirmed, further experimental settings will be focused on uPAR-CD36 interactions.

\textbf{b) Study on oxLDL binding by uPAR}

Zurück an: kruse.susanne@mh-hannover.de  
CD36 is a member of class B scavenger receptors binding oxLDL via its lipid moiety. As discussed in above, TLR sense modified lipids not via a typical receptor-ligand interaction but rather require cooperation with other receptors to elicit a pro-inflammatory response. This function might be served by CD36 \(^{21}\). It cannot be excluded, however, that uPAR, being a polyligand receptor \(^{22}\), possesses an ability to bind modified lipids, in particular oxLDL, most likely via its apoprotein moiety. To address this issue, we will first block oxLDL-CD36 binding using specific inhibitory peptide hexarelin. If this treatment would not affect the related functional outcome thus pointing to a possible uPAR involvement, further experiments on oxLDL-uPAR binding will be performed using Surface Plasmon Resonance technique (BIACore AB). Recombinant (R&D Systems) and/or purified (Americal Diagnostica) human uPAR will be used in these experiments. Functional activity and structural integrity of uPAR will be assessed in the preliminary experiments using its natural ligand uPA and specific binding antibody.

\textbf{c) Structural analysis of the signaling complex}

To verify nature and mechanisms of associations between uPAR, CD36, and TLR, several experimental settings on immunocytochemistry, immunoprecipitation, pull-down
assay, and chemical cross-linking will be performed. To analyse non-covalent, yet stable, protein interactions in living cells, fluorescence resonance energy transfer (FRET) will be used, as established and described by us 2,3. Finally, yeast two-hybrid system will be used to elucidate interacting proteins in vivo. The required techniques, plasmids, vectors, cDNA libraries, yeast culture and detailed protocols are available in the laboratory. The protein-protein interactions will be verified using the rescreen technique and the β-galaktosidase filter- and quantitative assays.

**Task 3. Elucidation of signaling pathways initiated by modified lipids via the uPAR-CD36-TLR interactome**

All three proteins of our interest, namely uPAR, CD36, and TLR are known to initiate multiple signaling cascades, though uPAR being a GPI-anchored receptor cannot signal directly and needs association with transmembrane proteins. Signaling capacities of CD36 and TLR also rely on cooperation with other receptors and formation of functional interactomes. We will therefore perform experiments aiming at elucidation, at least in part, of signaling pathways utilized in VSMC by uPAR-CD36-TLR association in response to modified lipids.

Based on our preliminary data and literature survey we suppose that the most probable candidates involved may be myocardin and NFκB pathways. A scenario might be suggested that oxLDL activates uPAR-myocardin axis leading to VSMC switch to a pathophysiologival phenotype. Association of uPAR with CD36/TLR may induce via their intracellular domains activation of NFκB signaling resulting in upregulation of pro-inflammatory mediators. If this mechanism could not be confirmed (though our preliminary data point to that), further candidates such as Jak-Stat, PI3-K, the Rho GTPases, c-Src, and Ras-Raf-MEK-ERK1/2 will be examined.

For biochemical experiments, association of signaling molecules and individual contribution of each of these interactions to cell functional changes and expression of proinflammatory mediators will be studied using experimental tools in hand in our laboratory. Nuclear signaling processes will be studied using experimental tools in hand in our laboratory. Myocardin and NFκB transcriptional activity will be examined using luciferase reporter assays.

Individual signaling pathways will be targeted using siRNA, expression of corresponding mutants and usage of inhibitors, and the functional outcome will be analysed as described in above. Final design of these experiments will be based on results of Task 1 and Task 2.

**Task 4. Studies on segregation of signaling proteins in lipid rafts as a regulatory mechanism**

Cell signaling does not occur randomly over the cell surface, but is integrated within cholesterol-enriched membrane domains, termed lipid rafts. A morphologically distinct subpopulation of lipid rafts is known as caveolae 27. Lipid rafts are thought to provide the infrastructure for bringing certain receptors and downstream signaling intermediators into proximity, permitting the formation of competent signaling assemblies 28, 29. Recent studies implicate lipid rafts in various disease processes including vascular disease 30, 31. Our recent findings suggest that location in lipid rafts may be a major factor in regulating the association of uPAR among multiple candidate partner proteins and intracellular signaling intermediates 6. Data on oxLDL impact on rafts are controversial and the underlying mechanisms are not understood yet 30. A growing body of evidence implicates segregation into lipid rafts as a mechanism controlling functional processes mediated via CD36 and TLR. In particular,
ligand specific heterotypic associations of TLR2/6 with CD36 and recruitment to lipid rafts in response to diacylated lipoproteins have been reported 21. To elucidate whether transient partitioning of uPAR, CD36, TLR, and related signaling molecules in rafts can regulate specific signaling directed by modified lipids, several experimental setting will be performed. Expression of fluorescently labeled uPAR in VSMC will be performed, as established, and specific constructs expressing fluorescently labeled other proteins of interest will be introduced by lentiviral infection or nucleofection. To analyse intermolecular interactions in living cells, FRET will be used. In parallel to FRET analysis, lipid rafts will be isolated using cell fractionation in sucrose gradient, as established. Fractions will be analyzed for proteins of interest using immunoblotting. Lipid rafts will be disrupted by cell treatment with drugs perturbing cholesterol pools, such as methyl-β-cyclodextran (MCD) and filipin or inhibiting cholesterol biosynthesis (statins). Additionally, caveolin-1 deficient cells will be used. Lipid raft visualization will be achieved by cell staining with FITC-conjugated cholera-toxin B subunit (CTB-FITC).

**Task 5. In vivo studies**

To verify functional relevance of *in vitro* findings, *in vivo* studies will be performed. Using a model of periadventitial application of modified lipids to carotid arteries in pluronic gel, we will examine the effects of oxLDL, mmLDL, and oxPL on induction of atherogenic inflammatory signals *in vivo*. Numerous studies have been performed on atherogenic effects of oxLDL *in vivo*. However, this research addressed mainly the advanced phase of atherosclerosis and correspondingly *ApoE−/−* mice have been used 32. In contrast to these studies, we are interested to elucidate whether and how modified lipids induce atherogenic inflammatory genes in arterial wall *in vivo* under conditions mimicking early steps of vascular disorder.

We will use 8-week old male C57BL/6 mice; 7 animals per group. Modified lipids will be used in concentrations, which are lower than the concentrations measured in atherosclerotic lesions of small animals. Immediately before surgical application, oxLDL, mmLDL, and oxPL will be dissolved in cold 1% (wt/vol) F-127 pluronic gel (Sigma, St Louis, MO) in sterile water, followed by addition of 5 volumes of 50% (wt/vol) F-127. Sixty microliters of pluronic gel with or without modified lipids will be applied to the left carotid arteries; application of plain gel to the right carotid arteries will be used as controls (sham). To consider on contribution of uPAR to inflammatory process, uPAR−/− mice on the C57BL/6 background (8-week old male, 7 animals per group) will be additionally used. For gene expression analysis of pro-inflammatory cytokines and chemokines, arteries will be removed from euthanized and PBS-perfused animals 24 hours after surgery. Tissue harvesting, RNA isolation, and RT-PCR will be performed as established and described 2. For comparison of gene expression levels between these animals and those with advanced atherosclerosis, *ApoE−/−* mice at 12 months of age will be used. To examine whether application of modified lipids may induced changes in cellular composition of arteries, immunocytochemical analysis of deep-frozen arteries cross sections will be performed as described 5.

**Funding:** DFG (DU 344/7-2, renewal proposal, submitted)

**Time schedule**

First year: Studies on cell functional and inflammatory properties; structure-functional studies on uPAR interactome.

Second year: Studies on signaling pathways and lipid rafts.

Third year: In vivo studies; writing the thesis.

**Group Members:**

Inna Dumler, Prof., PhD; Yulia Kiyan, PhD; Margret Patecki, MD; Sergey Tkachuk, PhD;
Natalia Tkachuk, PhD; Krishna Vallabhaneni, PhD student; Mahshid Hodjat, PhD student; Parnian Kalbasianaraki, PhD student; Birgit Habermeier, technician; Frank Hausadel, technician; Jan Thomas, technician; Petra Wübboldt-Lehmann, technician.

Key References for Project:

Zurück an: kruse.susanne@mh-hannover.de

Own references (mainly 2009-2012):