Final report

Leibniz Graduate School for Rheumatology

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Content

1 Executive summary

Understanding immunological memory and its role in rheumatic diseases and finding new ways to improve patient therapy demands a research environment in which experimental biomedical scientists work very closely together with clinical scientists. In the Leibniz Graduate School for Rheumatology (LGRh), medical doctoral students and PhD students from natural sciences have teamed up and addressed central aspects of the key question how memory cell survival is regulated. In the clinically oriented projects, biomarkers were identified in order to enable an optimal, individualized therapy, in the sense of personalized medicine.

Scientific highlights include:

- Human bone marrow memory CD4 T cells form a separate compartment, with their specificities enriched for systemic antigens. Upon vaccination, they can be rapidly mobilized into the blood circulation, and contribute to the immune response.
- The precursors of bone marrow memory CD4 T cells are CD49+T-bet+ CXCR3+ activated CD4 T cells, which are predominantly generated in the late phase of an immune response.
- In an approach to understand the functional impact of circular RNAs on T cells, next generation sequencing of pro-inflammatory CD4 T cells identified the circRNA Ikzf1to be expressed differentially in naive versus memory T cells, with possible impact on the regulation of chronically activated T helper subsets.
- In a SLE mouse model, it was shown for the first time that long lived plasma cells producing pathogenic autoantibodies are located in the intestinal lamina propria.
- In order to analyze the microenvironment of long lived plasma cells, MELC technology, a method for analyzing dozens of markers in one single histological section, was established and showed that B progenitor cells and plasma cells occupy distinct niches in the marrow.
- The finding that human innate immune cells can acquire trained immunity against viruses challenged the dogma that innate immunity does not contribute to immunological memory.
- Mass cytometry was established for human urine samples. This allowed the distinction of different types of kidney inflammation, based on biosignatures on immune cells.
- Novel renal autoantigens were identified in lupus patients, and the frequency of CD4+ T cells specific for these antigens in the blood was shown to correlate with the activity of lupus nephritis.
- The detection of urinary T cells by flow cytometry was found to be a marker for intrarenal inflammation after renal transplantation.
- The myeloid surface marker SIGLEC-1 was identified as a biomarker for an interferon alpha signature and proved to be a robust marker for active disease in patients with primary Sjögren´s syndrome and SLE. Moreover, high maternal expression of SIGLEC1 was shown to be a risk factor for the development of autoimmune congenital heart block.
- A novel bioinformatics approach was used to identify cytokine expression patterns characteristic for T cells in MS patients.

In the LGRh, the students gained a sound knowledge of immunological memory in healthy and diseased organisms and of various methods of studying the complex immune system. Moreover, they obtained soft skill training and mentoring. They presented their projects at numerous conferences and at the same time built up a network for their future careers. In October 2017 the doctoral students organized the 2-day symposium “LGRh/LeGCI Graduate Symposium - from basics to clinics”, at which invited guest speakers and doctoral students presented their projects.

The LGRh has closed a gap in the existing portfolio of graduate schools in Berlin regarding the aspect of interdisciplinary education in immunology and rheumatology. The research focus of graduate education at the DRFZ is now being extended from rheumatology to other chronic inflammatory diseases in the successor program Leibniz Graduate School on Chronic Inflammation, LeGCI.

2 Initial questions and objectives of the LGRh

The DRFZ has developed the concept of a pathogenic immunological memory driving chronic inflammation in rheumatic diseases. The LGRh is based on this research concept. Accordingly, the projects of the LGRh had the aim to investigate different aspects of immunological memory in healthy organisms, as well as in rheumatic inflammatory diseases. How is the immunological memory organized and where do the cells survive? Do the cells of the innate immune system also form a memory? Are there special characteristics of pathogenic memory cells that could serve as targets for new therapies? From a basic understanding of immunological memory, therapeutic strategies are developed at the DRFZ. Along that line, some of the projects followed the research rationale of personalized medicine. These projects aimed at determining biomarkers for diagnosis, disease monitoring and the measurement of therapeutic success. The focus was on the analysis of urine samples from patients with renal involvement, but also on the analysis of blood samples of patients with different autoimmune disorders.

Beyond the scientific project, the scope of the LGRh was to grant excellent educational conditions for doctoral students who wanted to become experts and future leaders in experimental and/or clinical rheumatology. Before the establishment of the LGRh, there was no structured doctoral training focusing on rheumatology in Berlin. Since research at the DRFZ is strongly translational, it was of central importance to include both science doctoral students and medical doctoral students in the LGRh; we explicitly wanted to support highly motivated medical doctoral students to conduct demanding experimental scientific projects that provide a basis for a successful career as clinical scientists. Thus, in addition to the 6 projects for PhD candidates with a funding duration of three years, we have included 6 positions for medical doctoral students in the program, each with a duration of one year. By this, also science doctoral students got a deeper insight into the clinical aspects of rheumatic diseases, in order to gain a better understanding of the challenges in patient treatment and to see the limits that still exist in treatment today. This is intended to motivate the young scientists to focus their future research career to these challenges.

3 Results

3.1 Structural measures of the graduate program

3.1.1 Establishment of the graduate school and recruitment of doctoral students

The LGRh started with the appointment of Dr. Katrin Moser as coordinator. She organized the announcement for the doctoral positions (see below) and prepared the internet presence of the LGRh on the DRFZ's website (no longer available online following a website relaunch in 2018; a brief summary of the LGRh can still be found on the new homepage (www.drfz.de)).

The LGRh was associated to the ZIBI Graduate School of the “Interdisciplinary Center for Infection Biology and Immunology” of Humboldt-University Berlin (HU) (https://www.zibi-berlin.de). ZIBI is the roof of several local graduate programs, bridging individual scientific and biomedical disciplines, such as immunology, virology and bacteriology, and is internationally recognized. This has decisively increased international visibility of the Leibniz graduate school. As a member of the ZIBI Graduate School, the LGRh doctoral researchers profited from the Humboldt Graduate School (HGS) and the Dahlem Research School (DRS), the umbrella organizations for structured doctoral programs of Humboldt University Berlin and the Free University, respectively. These programs offer a wide range of training opportunities primarily to promote transferable skills.

The PhD positions in the LGRh were advertised in relevant national and international forums (Naturejobs online, Laborjournal (German and European website and print)), on the DRFZ website, as well as spread by national and international cooperation partners. The students were selected in an assessment center. Of 35 applicants from 11 different nations, 14 were invited to the assessment center which took place on April 04 - 05, 2014. Within the first year all 6 PhD students started their projects. The 6 MD students started their one-year projects at various time points over

the 4-year funding phase. These students mostly performed their experimental work during a sabbatical period of their medical curriculum. LGRh funding enabled them to dedicate their time specifically to these projects. The medical doctoral students were mainly recruited via our liaison research groups with the Charité – University Medicine Berlin and were directly selected by the thesis committee of the respective project. Each doctoral student was supervised by a thesis committee consisting of three supervisors: the respective group leader, one scientist of a closely related topic and a third supervisor who provides advice and quality control. Ideally, one of the supervisors was a clinician to encourage the translational aspects.

3.1.2 Scientific education – retreats, workshops and Symposium

All doctoral students and supervisors met at least once a year at the LGRh retreat, which included the thesis committee meetings. The doctoral students presented the progress of their projects in talks and organized a poster session. The LGRh retreats took place on 08./09.06.2015, 15.07.2016, and on 09./10.10.2017 as part of the "LGRh/LeGCI Graduate Symposium - *from basics to clinics*". In addition, the doctoral students attended the annual retreats of the ZIBI Graduate School Berlin. Besides these annual meetings, the doctoral students attended the weekly scientific discussion clubs of the chronic inflammation forum at the DRFZ. Here, scientists meet at the DRFZ in an interdisciplinary and thematically focused manner and discuss ongoing research projects. This serves as a central measure for quality control and trains students to present and critically discuss their projects and to network.

In order to gain a sound knowledge of immunology, PhD students attended the Spring School on Immunology in Ettal, Bavaria, an advanced training on immunology organized by the German Society for Immunology (DGfI). Medical PhD students attended the DGfI Autumn School "Current Concepts in Immunology" which provides a solid basic knowledge in immunology, or the more clinically oriented Translational Immunology Schools (TIS) of DGfI.

The doctoral students actively took part in various scientific conferences, for personal training and to present their research results (see 3.3.2). In October 2017, the LGRh doctoral students organized the "LGRh/LeGCI Graduate Symposium - *from basics to clinics*". The PhD students compiled the scientific program with four sessions: *Bone Marrow*, *Memory*, *Translation into clinical application* and *Innate Immunology*. For each session, guest speakers were proposed by the doctoral students and the selection was then made in consultation with the speakers of the LGRh and the LeGCI, Anja Hauser and Helena Radbruch. The doctoral students invited the guest speakers, hosted them and chaired the respective sessions in which they also presented their own scientific project. The "LGRh/LeGCI Graduate Symposium - *from basics to clinics*" was the kick-off to the LGRh successor program Leibniz Graduate School on Chronic Inflammation (LeGCI, see sec. 3.4).

3.1.3 Job-Skill / soft skill training

In addition to the scientific training, the doctoral students received soft skill training in various courses: "Mastering your PhD, Managing your Projects" by Dr. Valeska Russo, proSciencia (24./25.11.2014); "Scientific Writing" by Dr. Avril Arthur-Goettig, bioxpress (18./19.07.016) with a focus on the preparation of scientific publications; "Statistics and GraphPad Prism 7" by Dr. Helmut Schlumprecht, Statistik-Service (27./28.10.2016); and "Writing Scientific Grant Proposals" by Andrea Sanchini, Ph.D., Sanchini Scientific Writing (27.10.2017). In addition to these workshops, doctoral students attended workshops offered by external providers, e.g. to deepen the knowledge about specific methods relevant for their individual research project.

3.2 Scientific reports of the doctoral projects

3.2.1 How is the immunological memory organized and where do the cells survive?

Function and compartmentalization of circulating versus tissue resident memory T cells

Carla Cendón, PhD project

Supervisors: Andreas Radbruch, Jun Dong, Falk Hiepe

Intensified efforts to promote protective T cell-based immunity in vaccines and immunotherapies have created a compelling need to expand our understanding of human T cell function and maintenance beyond its characterization in peripheral blood. Memory T cells reside in a variety of tissues, including the bone marrow (BM). However, the division of labor and the lifestyle of circulating versus tissue resident memory T cells is poorly understood. Bone marrow memory T cells are CD69+ and resting in terms of transcription profile, proliferation and migration. Since memory $CD4^+$ T cells specific for systemic childhood antigens, like measles, mumps or rubella have been found in the BM of elderly humans, even when they were no longer detectable in peripheral blood (PB) circulation, we hypothesized that BM memory T cells are resident, resting and maintain long- term memory to systemic antigens.

In this project we have shown that PB and BM memory T cells have different survival capacities, being the CD69+ BM memory T cell subset the one showing reduced half-life without any additional stimulus. Moreover, we identified the important role of IL-7, IL-15 and direct contact with stromal cells in memory T cell maintenance, either increasing the Bcl2/bim ratio or Mcl-1/Noxa ratio respectively. Moreover, using flow cytometric and sequencing analysis of the TCRβ repertoire, we have determined that PB and BM memory T cells are separated cell populations, highly compartmentalized in their respective tissues. Finally, by tracking the dynamics of antigen-specific memory CD4+ T cells after systemic MMR re-vaccination we could show that TRM CD4+ T cells specific for systemic antigens can be rapidly mobilized into blood circulation and contribute to the immune response.

Taken together, these studies provide a more comprehensive understanding of the function and maintenance of immunological memory in humans.

Deciphering the generation of bone marrow resident memory CD4 T cells in the spleen

Jana Sarkander, PhD project

Supervisors: Koji Tokoyoda, Anja Hauser, Falk Hiepe

Long-lived memory CD4 T lymphocytes play a crucial role in the generation, maintenance and reactivation of other memory lymphocytes. During an immune reaction, some antigen-experienced CD4 T cells relocate from secondary lymphoid organs (SLOs) to the bone marrow (BM) and reside and rest there as memory CD4 T cells. However, it remains elusive how the precursors of BM memory CD4 T cells are generated in SLOs.

The project aimed at the phenotypical identification and functional characterization of splenic antigen-specific memory CD4 T cell precursors that ultimately populate the memory pool in the BM. By means of adoptive transfer of TCR-transgenic CD4 T cells and immunization of cognate peptide, the first part of this project identified splenic CD49b+T-bet+(CXCR3+) activated CD4 T cells as the precursors of BM memory CD4 T cells. Subsequent to their identification, the second part highlighted that precursors of BM memory CD4 T cells are generated following enhanced cell proliferation and prolonged cognate interactions with dendritic cells (DCs) in the late activation phase of a primary immune response. Treatment with a cytostatic drug or blockage of CD28/B7 co-stimulatory pathway in the late activation phase in turn abrogated the generation of BM memory CD4 T cell precursors. Moreover, fluorescent-dye labeling experiments demonstrated that the more CD49b+CXCR3+ activated CD4 T cells divide, the more they lose the expression of CCR7, a chemokine receptor crucial for cell egress from the T cell zone of SLOs, and gain the expression of IL-2Rβ, a cytokine receptor crucial for long-term survival.

Since B cells have been previously described to modulate the formation of memory CD4 T cells in the spleen, the third part of the project investigated the role of B cells for the establishment of CD4 T cell memory in the BM by using B cell-depleted or B cell-deficient mice. B cells had a negative impact on the accumulation memory CD4 T cell precursors in the BM but did not affect the cell division of activated CD4 T cells in the spleen during the activation phase, raising further questions on the regulatory role of B cells.

In sum, the results obtained in this project provide new insight into the generation of BM resident memory CD4 T cells that may help for the therapeutic strengthening of memory in the context of vaccination or its abolishment within the scope of autoimmune diseases.

The role of circular RNAs in CD4 T helper cells

Cam Loan Tran, PhD project

Supervisors: Mir-Farzin Mashreghi, Andreas Radbruch, Joachim Sieper

Chronic autoimmune diseases such as Rheumatoid Arthritis are characterized by the perpetual inflammation of the joints caused by infiltrating immune cells that ultimately lead to the degradation of surrounding tissue. Hereby, pro-inflammatory CD4 T helper (Th) cells play a crucial role by initiating and maintaining the chronic status through the expression of particular genes that support their survival and expansion.

In this project, we set out to identify and characterize circular RNAs that could potentially play a role in the adaptation process of pro-inflammatory CD4 Th cells using Next-Generation-Sequencing. Among the > 40.000 potential circular RNAs identified in Th cells, we focused on the highly expressed circular Ikzf1 (circIkzf1), a molecule that is differentially expressed between naive and repeatedly reactivated Th cells. CircIkzf1 is a 174-nucleotide long, covalently closed RNA molecule which is formed upon head-to-tail splicing of exons 2 and 3. Interestingly, both of these exons are also part of the linear Ikzf1, leading us to the question whether the decision in favor of a circular RNA or mRNA molecule is made during the process of competitive splicing or competitive transcription. For that, a CRISPR-mediated gene activation system (dCas9-VP64) was used to target both alternative and conventional promoters of the Ikzf1 locus. As we found out, enhancing the alternative promoter-dependent transcription selectively increased circIkzf1 expression about 5-fold without altering Ikzf1 mRNA expression. Vice versa, inducing conventional promoter-dependent gene expression did not result in a significant induction of the linear mRNA but suppressed the circIkzf1 expression, indicating that circIkzf1 and mRNA Ikzf1 use different promoters and compete on a transcriptional level. In addition, RNA Fluorescence in situ hybridization assays allowed us to locate the circIkzf1 in the cytoplasm, in contrast to the chromatin-remodeling transcription factor Ikzf1.

Interestingly, ribosomal profiling revealed the loading of circIkzf1 onto polysomes, suggesting its translation into a truncated protein. This was furthermore validated by Western Blot, which also showed that translation of circIkzf1 resulted in the detection of several concatemers of different lengths. Since circIkzf1 contains a start- but no stop- codon with a sequence that is a multiple of 3, its translation can potentially be infinite, making the translation continuous which resulted in the detection of several protein products.

Currently, we are investigating the functional role of the truncated circIkzf1 derived protein and will continue to assess the role of our candidate in pro-inflammatory Th cells, thereby contributing to the basic understanding of the functional impact of circRNAs on T cells.

Lamina propria of the small intestine provides survival niches for memory plasma cells

Dominik Lammerding, MD project

Supervisors: Anja Hauser, Bimba Hoyer, Falk Hiepe

Autoreactive long-lived plasma cells (PCs) play a major role in the pathogenesis of systemic lupus erythematosus. Lymphatic organs such as spleen and bone marrow (BM), as well as inflamed organs such as inflamed kidneys harbor long-lived memory plasma cells (MPCs). These cells are refractory to most immunosuppressive therapies. In protective humoral memory, mucosal MPCs have been shown to play an important role.

In this project, the intestinal lamina propria in lupus prone NZB/W F1 mice was analyzed for the presence of long-lived PCs and if these would produce autoantibodies. To this end, 7 months old NZB/W mice were EdU-fed for 14 days in order to quantify the frequency and number of long-lived PCs in the lamina propria of the small intestine. Cells were analyzed by flow cytometry for the presence of PC markers (kappa light chain, CD138) and EdU incorporation. Elispot was used to detect the number of autoreactive (dsDNA-specific) PCs and the presence of autoreactive antibodies in cell culture supernatants was determined by ELISA.

We could show that about 80% of the intestinal PCs in aged NZB/W mice with symptomatic lupus nephritis are indeed long-lived MPCs. Using Elispot and ELISA we could show that the population of intestinal PCs included dsDNA- specific PCs, in the majority of IgA isotype.

It still needs to be shown if the autoreactive mucosal PCs themselves are also long-lived and if they contribute to systemic autoimmunity. Further analysis is also needed with regard to the present cytokine milieu and the role of neighboring cells. Nevertheless, the presence of this important MPC compartment has to be taken into account in considering treatment strategies and efficiency.

Analysis of stromal heterogeneity in the murine bone marrow using multiplexed fluorescence microscopy

Karolin Holzwarth, PhD project

Supervisors: Anja Hauser, Raluca Niesner, Thomas Dörner

The bone marrow (BM) is structured in multiple micro-environmental entities (niches), which maintain immunological memory and control of the hematopoietic stem cell pool as well the differentiation of hematopoietic cells. The so-called survival niches contain hematopoietic cells and stromal cells, which provide the longevity of memory plasma cells and memory TCs as well as of hematopoietic stem cells and B progenitors. However, due to the molecular and cellular complexity and a lack of suitable histological multiplexing methods, the composition of the various BM niches is still elusive. Technical and methodical advances in flow cytometry as well as improvements by *in situ* and *in vivo* images now enables the characterization of the network-like structured stroma, grouped in several fractions by phenotypic markers such as Paired-related homeobox 1, CXCL12, leptin receptor, nestin or interleucin 7. The aim of this work was to elucidate the heterogeneity of BM stromal cells as a crucial part of the survival niches.

Multi-epitope-ligand-cartography (MELC) was developed to histologically co-map dozens of different proteins (markers) in single tissue sections by automated imaging cycles. We applied MELC on bone sections from CXCL12-GFP mice. We combined multiplexed immunofluorescence histology data with various object-based segmentation approaches in order to define irregularly shaped, net-like structures of stromal cells. We confirmed MELC as a robust histological method and validate our automated segmentation algorithms using flow cytometry and manual evaluation. By means of MELC multiplexing, we revealed heterogeneous expression of leptin receptor, BP-1 and VCAM-1 within the stromal network. Moreover, we demonstrated by quantification a preferential contact of B cell subsets as well as of plasma cells to stromal processes compared to stromal somata.

Our approach is suitable for spatial analysis of complex tissue structures.

Peptide-specific recognition of human cytomegalovirus strains Controls the Activation and Expansion of Adaptive Natural Killer Cells

Quirin Hammer, PhD project

Supervisor: Chiara Romagnani, Max Löhning, Klaus Osterrieder

Natural killer (NK) cells are part of the innate immune system and combat virus-infected and tumor-transformed cells. Accumulating evidence suggests that certain NK cells display features classically attributed to the adaptive immune system. Particularly, in response to human cytomegalovirus (HCMV), a subset of NK cells expressing the activating receptor NKG2C specifically proliferates and expands, which partly resembles pathogen-specific adaptive immune responses. Although the adaptive behavior of NK cells is being extensively studied, key aspects of adaptive NK cell biology require further investigation: (1) detection of adaptive NK cells remains challenging because their phenotype partly overlaps with conventional, non-adaptive NK cells, (2) adaptive NK cells display efficient effector responses against target cells, but are surprisingly poorly responsive to exogenous pro-inflammatory cytokines, and (3) although adaptive NK cells are evidently associated with HCMV infection, the population is not present in all, but only some HCMV-seropositive healthy individuals and the signals driving these heterogeneous adaptive NK cell responses remain unknown.

To address these questions, the aims of this project were (1) to explore whether co-expression analysis of previously described markers of adaptive NK cells can be employed to reliably detect the population, (2) to functionally interrogate whether adaptive NK cells have completely lost their ability to sense exogenous cytokine signals or whether they can integrate cytokine cues into the defined context of target cell encounter, and (3) to investigate whether polymorphic HCMV-derived ligands can contribute to the variable expansion and differentiation of adaptive NK cells in HCMV-seropositive donors. Integration of adaptive NK cell markers into a phenotypic signature enabled reliable detection of adaptive NK cells and receptor co-expression analysis clearly distinguished adaptive from conventional NK cell populations. Moreover, functional assessment revealed that adaptive NK cells retain responsiveness to the cytokine interleukin-18, which functions as a co-stimulatory signal during activation elicited by target cells. Furthermore, this work exposed that adaptive NK cells can utilize the germ line-encoded receptor NKG2C to differentially recognize distinct HCMV strains based on variable, HLA-E-binding peptides. In combination with pro-inflammatory signals, viral peptides control the expansion and the differentiation of adaptive NK cells. Thus, polymorphic HCMV peptides constitute a signal driving adaptive NK cell responses and shaping the heterogeneity of adaptive NK cell populations among HCMV-infected individuals.

Collectively, this project further delineates the biology of adaptive NK cells in terms of their function, generation, and heterogeneity.

3.2.2 Disease monitoring and biomarkers

Lupus Nephritis: investigating the urinary immune cells signature by mass spectrometry

Martina Bertolo, MD project

Supervisors: Andreas Grützkau, Philipp Enghard, Falk Hiepe

Currently the diagnosis of Lupus Nephritis (LN) is determined by renal biopsy and there are no good non-invasive parameters to substitute renal biopsy, allow monitoring of treatment response or predict prognosis. Urinary T cells have previously been reported to be a promising biomarker for LN. Here we present a comprehensive analysis of the immune cells present in the urine of patients with LN using mass cytometry, which enables the simultaneous measurement of over 30 markers/cell and hence the investigation of all major leucocyte populations.

Mass cytometry analysis was performed on urine and peripheral blood (PB) samples of 15 patients with a current kidney biopsy showing LN and no therapy changes within 4 weeks. Clinical and laboratory data were collected at the time of sampling and up to 6 months after treatment initiation.

The largest populations in urine were represented by neutrophils (with a mean of 42% of all cells, vs 81% in PB), macrophages (24% vs. 5%) and T cells (2% vs. 13%). B cells were less than 1%, eosinophils basophils NKs and mDCs were less represented and variably detectable.

The distribution of these major populations and of further characterized urinary cell subsets allowed the distinction of proliferative LNs from the membranous and the atypical LNs by an automated cluster analysis. Urinary macrophages showed mostly a classic inflammatory phenotype and expressed Siglec-1 similarly to PB monocytes. In the lymphocyte compartment, comparison of urine and PB showed a significantly lower CD4/CD8-ratio in urine than in PB. While naïve T cells prevailed in PB, effector memory (EM) T cells were the dominant subset in urine and had significantly higher expression of activation markers (CD69 and CD38) in comparison to EM T cells in PB. The percentage of activated EM T helper cells in urine correlated positively with the renal disease activity (SLEDAI). Patients with lower percentages of EM T helper cells in urine had better response to induction therapy six months after its initiation.

Urine and blood leucocyte populations differ considerably, supporting the theory that urinary cells derive from kidney-resident cells. The urinary “biosignature” consisting of variable leucocyte subsets´ constellations might allow a rough estimation of kidney pathology in LN. The presence of effector memory T cells in urine may be used to predict response to treatment.

The anti-renal CD4+ T cell response in human lupus nephritis

Sebastian Tesch, MD project

Supervisors: Gabi Riemekasten, Philipp Enghard, Falk Hiepe, Anja Hauser

Lupus nephritis (LN) is one of the most serious complications of the autoimmune disease systemic lupus erythematosus (SLE). A T cell rich infiltration in the kidneys, local MHC II upregulation and the identification of T cells in urine of LN patients suggests an antigen-specific immune response against kidney structures. However, at present no renal antigens are known for this disease.

Applying an algorithm in which we put together two assumptions, we were able to identify five candidate renal autoantigens for this project. Firstly, we assumed that a corresponding autoantibody is likely to be present in active LN and secondly that the respective target-antigen is upregulated in the inflamed kidney of LN patients. Undertaking a thorough literature research we identified potential autoantibodies. The expression levels of the respective targets were then retrieved from the GEO database. To test the CD4+ T cell reactivity with these potential renal autoantigens we used flow cytometry and T cell libraries. CD4+ T helper cells were stimulated with an antigen-pool that contained all five antigens. Cells expressing the activation marker CD154 were magnetically enriched and intracellular cytokine production was measured by flow cytometry. T cells then were enriched for CD137, which can be used to identify reactive regulatory T cells (Tregs). For the T cell libraries, cells were expanded for two weeks and then stimulated with single antigens. Their [3H]-Thymidin incorporation was measured in order to determine autoreactive responses. Stimulating with only one antigen, the experimental set-up was also successfully used for three cultures of urinary T cells from active LN patients.

No autoreactive CD4+ T cells were observed in patients with inactive SLE and in healthy controls upon stimulation with an antigen-pool. In contrast, in SLE patients with active LN, renal antigen reactive CD4+ T cells were significantly expanded. These cells were mainly IFN-γ producing Th1 CD4+ T cells. Their frequencies also correlated with disease activity. No significant expansion of renal autoantigen reactive Tregs was detectable in active disease. However, when calculating the ratio of autoantigen reactive IFN-γ+ Tcon cells to CD137+ Tregs, we observed a significant increase in active LN patients and this Tcon/Treg ratio correlated with disease activity.Using T cell libraries we finally were able to delineate the reactivity against the single used renal antigens. For

two antigens significantly more reactivity in active LN patients could be found in T cell libraries. Furthermore all three urinary T cell libraries showed reactivity to the probed antigen.

Altogether we were able to identify CD4+ T cells reactive with a pool of five proteins with increased expression in LN. These T helper cells were detected in higher frequencies in blood of SLE patients with an active renal disease compared to inactive patients or healthy individuals. Thus, a potentially pathogenic role for these cells in kidney inflammation can be discussed. Furthermore, a pathogenic imbalance is indicated by the observed shifts in the ratio of autoantigen-specific conventional to regulatory T cells.

Further experiments are still ongoing, but first results hint at the occurrence of autoantigen specific selfreactive T cells in the urine of active LN patients. These findings could further strengthen the concept of a pathogenic role of these autoreactive T helper cells in the course of LN development in humans.

Combination of urinary T cells, podocytes and tubular epithelial cells to detect renal transplant rejection

Hannah Brand, MD project

Supervisors: Andreas Grützkau, Philipp Enghard, Falk Hiepe

Currently there is a lack of non-invasive biomarkers to detect renal transplant (NTX) rejection. In this study we probed the hypothesis that the amount of podocalyxin (PDX)+ cells (as surrogate for podocytes), tubular epithelial cells (TEC) and T cells would reflect the extend of glomerular damage, tubular injury and inflammation respectively.

In this project, urine samples of 64 NTX patients were analyzed using flow cytometry. NTX patients with graft deterioration were categorized according to the corresponding renal biopsy result as having acute cellular rejection (ACR), humoral rejection (HRX) or no rejection (No RX). An additional cohort of NTX patients with stable graft function was included as control group. Urinary cells were stained for podocalyxin (surrogate for podocytes), Cytokeratin, CD10 and EPCAM (proximal and distal TEC) and CD3, CD4 and CD8 (T cells). Urinary T cell counts correlated with intrarenal inflammation. ACR patients had significantly more urinary T cells than patients with HRX or stable graft function, however the difference to patients with graft deterioration without rejection was not significant. Urinary PDX+ cells and TEC did not correlate with intrarenal glomerular or tubular damage, and unexpected high amounts of PDX+ cells and TEC were observed in the urine of patients without rejection. Calculating the amount of T cells per PDX+ cell and T cells per TEC delineated patients with acute rejection from patients without rejection and may be a potential biomarker for non-invasive detection of NTX rejection.

From our results we conclude that, while urinary T cells reflect intrarenal inflammation, urinary PDX+ cells (podocytes) and TEC seem not to directly reflect damage in the respective compartments of the kidney.

The application of the interferon surrogate parameter SIGLEC1 in a clinical setting

Anna Lisney, MD project

Supervisors: Thomas Dörner, Gerd-Rüdiger Burmester, Andreas Grützkau

Interferon-alpha (IFN-α) is a central cytokine in the pathogenesis of various autoimmune diseases, including systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS). As there are no standardized methods for directly measuring IFN-α, the expression of the myeloid-cell surface receptor SIGLEC1 was studied as a potential biomarker for an IFN-α signature. While previous studies found that SIGLEC1 correlated with disease activity in patients with SLE, the present study aimed to study SIGLEC1 in patients with pSS, where IFN-α is also known to play a central role. Additionally, we wanted to study SIGLEC1 and IFN-α in females whose children

developed autoimmune congenital heart block (CHB). CHB development is pathogenetically related to pSS and SLE, but no research has been performed investigating a potential role of IFN-α in this disease.

Within this study, we included 31 patients with pSS and evaluated the disease activity by ESSDAI score. We found that SIGLEC1 differed significantly between pSS patients with a disease manifestation that was restricted to the glandular organs (n = 15), and patients with a systemic disease manifestation (n = 16; p = 0.0001; Mann-Whitney-U test). This indicates that SIGLEC1 may be a valuable biomarker indicative of a more severe disease manifestation in patients with pSS, thus identifying patients potentially requiring more aggressive treatment.

We also analysed 9 mothers whose children developed CHB and compared these to 14 pregnant females whose children were exposed to anti-Ro antibodies in utero, thereby putting them at risk of CHB-development, and to 30 healthy pregnant females without anti-Ro antibodies. Here, we found that mothers whose children developed CHB had significantly higher levels of SIGLEC1 (p = 0.0034; MWU) and of IFN-α measured by ELISA (p = 0.0135; MWU), compared to the at-risk females who bore healthy children. Healthy pregnant women without the respective antibodies had the lowest levels of SIGLEC1 and of IFN-α. Therefore, SIGLEC1 may potentially be used for risk-stratification in pregnant females with anti-Ro antibodies.

In SLE, pSS and in females with a CHB pregnancy complication, expression of SIGLEC1 was reduced upon introduction of hydroxychloroquine and of oral glucocorticoids. This suggests that SIGLEC1 may be useful for dose titration and for monitoring treatment response in these diseases. In summary, this study was able to expand the knowledge on SIGLEC1 and IFN-α in pSS and CHB, and adds important new information for a potential clinical application of this biomarker.

Cytokine Patterns of T helper cells in Healthy Donors and Patients with Multiple Sclerosis

Ariana Mekonnen, MD project

Supervisors: Ria Baumgrass, Friedemann Paul, Andreas Hutloff

The activation of autoreactive T helper cells plays an essential role in the pathogenesis of demyelinating chronic autoimmune diseases, such as Multiple Sclerosis. Uncovering molecular mechanisms of these cells by analyzing cytokine patterns and surface activation markers therefore provides an important insight into the activated T cell repertoire and consequently helps to unveil auto-immunological mechanisms.

A subset of CD20+ T cells was identified that showed increased cytokine production and features of activation like high expression of PD-1 surface receptor indicating polyfunctional capacities. Complex relationships in the modulation of cytokine production were displayed with the help of a novel bioinformatics approach for pattern recognition of immune cells (PRI) developed in our group. Among cytokine co-producing memory T cells distinct patterns were observed, i.e. IFN-ɣ double-producing T helper cell were shown to never coproduce GMCSF and IL-21 simultaneously while IL-21 production was also correlated with PD 1 upregulation/activation.

PBMCs obtained from 16 Healthy Donors and 20 MS patients showed similar frequencies of circulating memory subpopulations between MS patients and healthy individuals. Multiparametric analysis of flow cytometric data revealed little differences between frequencies of single, double or multi-cytokine producers between patients and healthy individuals, with IL-10 producing effector memory T cells slightly elevated in MS patients.

We demonstrated the occurrence of a variety of complex T helper subsets with memory and activation features and propose PRI as a viable tool to characterize these populations. We found that in RR-MS patients with low-active disease in remission frequencies of activated, cytokine-producing memory T cells in peripheral blood are similar to those of healthy patients. We have shown that our approach contributes to the understanding of polyfunctional T-cell subsets, which will enable promising future applications in the investigation of multifunctional subsets and subsequently their application in clinical contexts.

3.3 Publications

3.3.1 Publications in peer reviewed original articles

Hammer Q, Rückert T, Romagnani C. Natural killer cell specificity for viral infections. Nat Immunol. 2018 Aug;19(8):800-808. doi: 10.1038/s41590-018-0163-6. Epub 2018 Jul 19.

Hammer Q, Rückert T, Borst EM, Dunst J, Haubner A, Durek P, Heinrich F, Gasparoni G, Babic M, Tomic A, Pietra G, Nienen M, Blau IW, Hofmann J, Na IK, Prinz I, Koenecke C, Hemmati P, Babel N, Arnold R, Walter J, Thurley K, Mashreghi MF, Messerle M, Romagnani C. Peptide-specific recognition of human cytomegalovirus strains controls adaptive natural killer cells. Nat Immunol. 2018 May;19(5):453-463. doi: 10.1038/s41590-018-0082-6. Epub 2018 Apr 9.

Hammer Q, Rückert T, Dunst J, Romagnani C. Adaptive Natural Killer Cells Integrate Interleukin-18 during Target-Cell Encounter. Front Immunol. 2018 Jan 17;8:1976. doi: 10.3389/fimmu.2017.01976. eCollection 2017.

Hammer Q, Romagnani C. About Training and Memory: NK-Cell Adaptation to Viral Infections. Adv Immunol. 2017;133:171-207. doi: 10.1016/bs.ai.2016.10.001. Epub 2016 Nov 30.

Holzwarth K, Köhler R, Philipsen L, Tokoyoda K, Ladyhina V, Wählby C, Niesner RA, Hauser AE. Multiplexed fluorescence microscopy reveals heterogeneity among stromal cells in mouse bone marrow sections. Cytometry A. 2018 Jul;93(9):876-888. doi: 10.1002/cyto.a.23526. Epub 2018 Aug 14.

Lisney AR, Szelinski F, Reiter K, Burmester GR, Rose T, Dorner T. High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block. Ann Rheum Dis. 2017;76(8):1476-80.

Rose T, Szelinski F, **Lisney A**, Reiter K, Fleischer SJ, Burmester GR, et al. SIGLEC1 is a biomarker of disease activity and indicates extraglandular manifestation in primary Sjögren's syndrome. RMD Open. 2016;2(2):e000292.

Sarkander J, Hojyo S, Tokoyoda K. Vaccination to gain humoral immune memory. Clin Transl Immunology. 2016 Dec 23;5(12):e120. doi: 10.1038/cti.2016.81. eCollection 2016 Dec.

Cendón C et al. Systemic immune re-challenge mobilizes functional human tissue-resident memory CD4+ T lymphocytes. *Manuscript in preparation*

3.3.2 Scientific presentations

10.-12.09.2014: Quirin Hammer: "Cytomegalovirus Drives Epigenetic Imprinting of the IFNG Locus in expanded CD94/NKG2Chi NK Cells" (poster); Natural Killer Cell Symposium 2014 of the DGfI Research Focus Group “Natural Killer Cells”, Hannover

31.10./01.11.2014 Sarnai Naran: poster, Meeting “Immunology of Mucosa”

12.-13.03.2015 Quirin Hammer: "Investigating the regulation of adaptive features in Innate Lymphoid Cells" (poster); ZIBI retreat 2015

12.03.2015 Karolin Holzwarth: “Histological multiplex analysis of plasma cell survival niches in the bone marrow” (poster); ZIBI Retreat 2015

12.03.2015 Cam Loan Tran: “Identification of circular RNA” (talk); ZIBI Retreat 2015

02.-06.05.2015: Quirin Hammer: "Cytomegalovirus Drives Epigenetic Imprinting of the IFNG Locus in expanded NKG2Chi NK Cells" (poster); “15th Meeting of the Society for Natural Immunity”, Montebello, Canada

06.-09.09.2015 Carla Cendón: “Maintenance of resting versus circulating memory T cells in humans” (poster); 4th European Congress of Immunology, Vienna, Austria

24./25.09.2015 Karolin Holzwarth: “Histological multiplex analysis of plasma cell survival niches in the bone marrow” (talk + poster); Leibniz Doktorandensymposium Sektion C

24./25.09.2015 Sarnai Naran: poster, Leibniz Doktorandensymposium Sektion C

04.-09.10.2015 Sebastian Tesch: “T cell autoreactivity in human lupus nephritis” (poster); 7th Autumn School Current Concepts in Immunology of the DGfI, Merseburg

07.-09.10.2015: Quirin Hammer: "Epigenetic imprinting regulates IFN-γ production in expanded NKG2C+ NK cells" (talk); Natural Killer Cell Symposium 2014 of the DGfI Research Focus Group “Natural Killer Cells”, Göttingen

07.-09.10.2015: Carla Cendón: “Resident versus circulating memory T cells in Humans” (poster); 25th Annual Meeting of the German Society for Cytometry, Berlin

07.-09.10.2015 Karolin Holzwarth: “Histological multiplex analysis of the spatial complexity of memory plasma cell microenvironments in the bone marrow” (poster); 25th Annual Meeting of the German Society for Cytometry, Berlin

13.10.2015 Quirin Hammer: "Peptide-specific recognition drives adaptive features in NK cells" (talk); DRFZ Labseminar

04.11.2015 Karolin Holzwarth: “Analysis of bone marrow microenvironments by MELC” (talk); MACS Club, Berlin

28.02.-04.03.2016 Carla Cendón: “Investigating the function and compartmentalization of circulating versus tissue resident memory T cells” (poster); 12th Spring School on Immunology, Ettal, Germany

28.02.-04.03.2016 Cam Loan Tran: “Identification of circular RNA” (poster); 12th Spring School on Immunology, Ettal, Germany

17.-18.03.2016 Quirin Hammer: "Peptide-specific recognition drives adaptive features in NK cells" (poster); ZIBI retreat 2016

17./18.03.2016 Karolin Holzwarth: “Histological multiplex analysis of the spatial complexity of memory plasma cell microenviroments in the bone marrow" (talk); ZIBI retreat 2016

14.-17.04.2016: Sebastian Tesch: “T cell autoreactivity in human lupus nephritis” (poster); ISN Nexus Symposium “Translational Immunology and Kidney Disease”, Berlin

14.06.2016 Cam Loan Tran: “Do Circular RNA control the Chromatin State in T helper cells?” (talk); DRFZ Labseminar

10.-13.08.2016: Sebastian Tesch: “The antirenal autoreactive CD4+ T cell response in human lupus nephritis” (poster); Kongress für Nephrologie Berlin

31.08.-03.09.2016 Dominik Lammerding: „Lamina propria of the small intestine provides survival niches for memory plasma cells” (poster); 44. Kongress der Deutschen Gesellschaft für Rheumatologie (DGRh), Frankfurt am Main

28.09.-01.10.2016 Hannah Brand: “FLOMOTX - Flow Cytometric Monitoring of Kidney Transplant Patients” (poster); European Student’s Conference Berlin

02.-05.10.2016: Quirin Hammer: "Adaptive NKG2C+ NK cells display antigen specificity for HCMV strains" (talk); “16th Meeting of the Society for Natural Immunity”, Taormina, Italy

05.-07.10.2016 Carla Cendón: “Resident versus circulating memory T cells in humans” (poster); 26th Annual Conference of the German Society for Cytometry (DGfZ)

27/28.02.2017 Sarnai Naran: poster, 2nd International Symposium Healthy Ageing, Magdeburg

09.-11.03.2017: Quirin Hammer: "Peptide-Specific Recognition of Human Cytomegalovirus by Adaptive Natural Killer Cells" (poster); Natural Killer Cell Symposium 2014 of the DGfI Research Focus Group “Natural Killer Cells”, Düsseldorf

16./17.03.2017 Cam Loan Tran: “Circular RNA expressed in T Helper Lymphocytes” (poster); ZIBI Retreat 2017

16./17.03.2017 Jana Sarkander: “The precursors of bone marrow memory T helper cells” (poster); ZIBI Retreat 2017

21.-25.04.2017 Hannah Brand: “Flow Cytometric Monitoring of Urine among Kidney Transplant Patients” (talk + poster), ISN World Congress of Nephrology, Mexico City, Mexico

25.04.2017 Quirin Hammer: "Regulation of Innate Lymphoid Cell Functions" (talk); DRFZ Labseminar

14.-17.06.2017 Anna Lisney: “High maternal expression of SIGLEC1 on CD14+ monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block” (talk); European Congress of Rheumatology EULAR, Madrid, Spain

20.06.2017 Karolin Holzwarth: “Histological multiplex analysis of the spatial complexity of microenvironments in the bone marrow” (talk); DRFZ Labseminar

12.-15.09.2017 Carla Cendón: “Mobilization of human tissue-resident memory T cells in response to systemic re-challenges” (poster); 47th Annual Meeting of the German Society for Immunology, Erlangen

12.-15.09.2017: Quirin Hammer: "Peptide-Specific Recognition of Human Cytomegalovirus by Adaptive Natural Killer Cells" (talk); 47th Annual Meeting of the German Society for Immunology, Erlangen

12.-15.09.2017 Jana Sarkander: “Generation of resting memory T helper cells following accelerated cell division” (poster); 47th Annual Meeting of the German Society for Immunology, Erlangen

12.-15.09.2017: Sebastian Tesch: “Antirenal CD4+ T cells arise in lupus nephritis, are mainly of the Th1 phenotype, are only partially controlled by their regulatory counterparts and invade the inflamed kidneys.” (poster); 47th Annual Meeting of the German Society for Immunology, Erlangen

09./10.10.2017 LGRh/LeGCI Graduate Forum - *from basics to clinics*, Berlin:

Carla Cendón: “Function and compartmentalization of circulating versus tissue resident memory T cells”

Quirin Hammer: “Peptide-specific recognition of human cytomegalovirus strains Controls the Activation and Expansion of Adaptive Natural Killer Cells”

Karolin Holzwarth: “Histological multiplex analysis of plasma cell survival niches”

Anna Lisney: “The application of the interferon surrogate parameter SIGLEC1 in a clinical setting”

Ariana Mekonen: “Cytokine patterns of T cells in patients with neuroinflammatory autoimmune diseases”

Sarnai Naran: “Depletion of plasma cells by targeting proteasome system”

Jana Sarkander: “Generation of resting memory T helper cells following accelerated cell division”

Sebastian Tesch: “The anti-renal CD4 T cell response in human lupus nephritis”

Cam Loan Tran: “Circular RNA Ikzf1 is expressed in T helper cells and can be translated into a cryptic protein”

22.-24.10.2017 Sebastian Tesch: “Antirenal CD4+ T cells arise in LN, are mainly of the Th1 phenotype, are only partially controlled by their regulatory counterparts and infiltrate the kidneys” (talk); Human Immunity 2017, Banff, Canada

19.12.2017 Cam Loan Tran: “Circular RNA Ikzf1 is expressed in T helper cells and can be translated into a cryptic protein” (talk); DRFZ Labseminar

12.04.2018 Cam Loan Tran: “The Role of Circular RNA in T Helper Lymphocytes” (poster); 4th Non-coding RNA & Epigenetic Regulation in Immune Cells, Berlin

12./13.04.2018 Karolin Holzwarth: “Multiplexed fluorescence microscopy reveals heterogeneity among stromal cells in mouse bone marrow sections” (talk); ZIBI retreat 2018

14.-17.04.2018 Dominik Lammerding: “Lamina propria of the small intestine provides survival niches for memory plasma cells” (talk); 124th Congress of the German Society for Internal Medicine, Mannheim

02.-05.09.2018 Cam Loan Tran: “Circular RNAs are abundantly expressed in a subset- specific manner in T helper lymphocytes” (poster); 5th European Congress of Immunology, Amsterdam, Netherlands

3.3.3 Completed doctoral qualifications

Carla Cendón: "Function and compartmentalization of circulating versus tissue resident memory T cells", Humboldt University Berlin, 2018

Quirin Hammer: "Regulation of adaptive natural killer cell functions and heterogeneity"; Humboldt University Berlin, 2018

Jana Sarkander: “Deciphering the generation of bone marrow resident CD4 T cells in the spleen”, Humboldt University Berlin (submitted 2018)

Anna Lisney: "Analysis of SIGLEC1 as a surrogate marker for a type I interferon signature in autoimmune congenital heart block and primary Sjögren's syndrome", Charité University Medicine Berlin (submitted 2017)

The other doctoral students or are in the final stages of their work, except for one doctoral student who has abandoned the doctoral thesis for personal reasons.

3.3.4 Awards

Quirin Hammer: travel award by Society for Natural Immunity, “15th Meeting of the Society for Natural Immunity”, 02.-06.05.2015, Montebello, Canada

Carla Cendón: poster prize, 4th European Congress of Immunology, 06.-09.09.2015, Vienna, Austria

Quirin Hammer: travel award by German Society for Immunology & travel award by Miltenyi Biotec, “16th Meeting of the Society for Natural Immunity”, 02.-05.10.2016, Taormina, Italy

Hannah Brand: “Young Nephrologists Best Clinical Abstract” and travel award, ISN World Congress of Nephrology, 21.-25.04.2017,Mexico City, Mexico

Anna Lisney: "EULAR Undergraduate Abstract Award“ and travel award; European Congress of Rheumatology EULAR, 14.-17.06.2017, Madrid, Spain

Dominik Lammerding: second price “Young Investigator Award”; 124th Congress of the German Society for Internal Medicine, 14.-17.04.2018, Mannheim

Quirin Hammer: Avrion Mitchison Award 2018 of the Ernst Schering Foundation for the best experimental, clinical or epidemiological research in the field of chronic inflammation

Holzwarth K, Cytometry A. 2018: “Paper of the month”, chosen for a “CYTO U” webinar of ISAC´s CYTO University

3.4 LGRh successor program - LeGCI

To continue structured education of doctoral students at the DRFZ, the successor program LeGCI was kicked-off at the “LGRh/LeGCI Graduate Forum - *from basics to clinics*”, October 9-10, 2017. LeGCI is the graduate program of the Leibniz ScienceCampus on Chronic Inflammation and is open to all PhD- and MD students of the DRFZ, as well as for students of the partner institutions within the Leibniz ScienceCampus on Chronic Inflammation.