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MDC MAX DELBRÜCK CENTER FOR MOLECULAR MEDICINE IN THE HELMHOLTZ ASSOCIATION

Antibodies & Complement Conference - May 20-25, 2019

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Abstract No. P1

Complement-enhancing monoclonal antibodies as a therapeutic strategy against Streptococcus pneumoniae

Leire Aguinagalde[1,*], *Suzanne M. Castenmiller*[1], *Carla J.C. Gosselaar-de Haas*[1], *Piet C. Aerts*[1], *Kok P.M. van Kessel*[1], and *Suzan H.M. Rooijakkers*[1]

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Streptococcus *pneumoniae* is a leading cause of community-acquired pneumonia, bacteraemia and meningitis. Current vaccines against pneumococcal infections are efficient for preventing invasive disease. However, the increased serotype replacement and antibiotic resistance, reinforces the necessity of developing alternative treatment strategies against *S. pneumoniae*. Monoclonal antibodies that boost the host immune system are attractive candidates to fight the high rates of morbidity and mortality due to this important human pathogen. Antibody-based immune activation can be induced by antibodies that bind bacterial surface structures and subsequently activate the complement cascade. Complement activation results in deposition of complement factors onto bacterial surfaces that mediate phagocytosis and intracellular killing by phagocytic immune cells. Recent studies have shown that specific point mutations in the Fc domain can enhance the clustering of antibodies into hexameric structures that are required for complement activation.

This study examines the efficacy of capsule-specific human monoclonal antibodies against *S. pneumoniae* serogroup-6. Using flow cytometry, we show that monoclonal antibodies harboring the hexamer-enhancing E430G or E345K mutations potently increase complement activation and phagocytosis of *S. pneumoniae* serotype 6B. Bacterial killing assays demonstrate the strong potency of engineered antibodies to induce neutrophil-dependent killing of *S. pneumoniae*. Furthermore, the cross-specificity found with pneumococcal serogroup-19 broadens the efficacy of the hexamer variants to protect against highly invasive *S. pneumoniae* isolates or against capsular polysaccharides that currently show unsuccessful protection in vaccines.

This work represents a first systematic approach to design effective therapeutic antibodies against *S. pneumoniae* with increased potency to activate the human immune system.

Abstract No. P2

Identifying functional antibodies that protect pregnant women against placental malaria infection

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Plasmodium falciparum malaria is caused by a parasite which regularly switches the antigens it expresses on the surface of the infected red blood cell (IRBC). An effective vaccine is required to reduce the morbidity and mortality caused by malaria, but the characteristics of an effective vaccine induced antibody-response remain unknown. The antigenic diversity of the parasite makes measuring protective antibodies difficult, as is distinguishing antibody responses that indicate exposure from those that indicate protection. Pregnant women are especially susceptible to malaria, because the parasite can express a specific protein, called VAR2CSA, on the IRBC surface which mediates placental sequestration. We use the unique correspondence between a single parasite antigen expressed and the specific pathology of placental parasitemia to try and identify protective antibody responses.

Method: We performed a case control study in 127 pregnant Papua New Guinean women, grouped into non-infected, infected with placental malaria, and infected with non-placental malaria based on placental histology, peripheral blood smear and PCR results at delivery. We examined a broad array of antibody responses towards VAR2CSA expressed on IRBC using plasma collected in mid-pregnancy.

Results: In mid-pregnancy, women who had malaria without placental infection at delivery had significantly higher levels of IgG1 and IgG3 ($P<0.05$) (but not IgG2 nor IgG4) towards the surface of the placental binding, VAR2CSA-expressing IRBC compared to women who had placental malaria. Levels of antibody dependent phagocytosis by THP-1 monocyte-like cells in plasma and in purified IgG were also higher in these women ($P=0.0002$ and $P=0.02$ respectively). We further measured opsonic antibodies using beads coated with individual domains of VAR2CSA protein but found no significant associations with protection ($P>0.05$). We are currently examining additional functional predictors of immunity including antibody dependent neutrophil reactive oxygen species production.

Conclusion: Antibodies (likely IgG1 and IgG3) that promote phagocytosis of IRBC are associated with protection from placental malaria andcould play an important role in protection from blood stage malaria infections in general.

Abstract No. P3

The anti-IgE antibody Omalizumab can induce adverse reactions through engagement of Fc gamma receptors

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IgE antibodies are key mediators of allergic diseases. Upon exposure to an allergen in allergic patient, the allergen is recognized by specific IgE bound to their high affinity receptor FceRI on the surface of mast cells, basophils and other effector cells, leading to the release of histamine and other mediators responsible for allergic symptoms. Omalizumab (Xolair) is an anti-IgE IgG1 monoclonal antibody (mAb) approved for the treatment of severe asthma and chronic spontaneous urticaria (CSU), and shows promises for treatment of other allergic diseases. Use of Omalizumab is associated with reported side effects, ranging from local skin inflammation at the injection site to systemic anaphylaxis. To date, the mechanisms through which Omalizumab induces adverse reactions are still unknown. Based on our finding that IgG-immune complexes (ICs) can induce anaphylaxis (Balbino et al., JACI 2017), we hypothesized that the side effects of Omalizumab could be due, at least in part, to activation of Fcγ Receptors (FcgR) by Omalizumab/IgE ICs. To address this, we demonstrated that ICs formed between Omalizumab and IgE can activate human neutrophils *ex vivo*. Additionally, these ICs can induce both skin inflammation and anaphylaxis through engagement of IgG receptors (FcgRs) in FcgR-humanized mice (expressing hFcγRI, IIA, IIB, IIIA & IIIB in place of all mouse FcγRs). We further developed an Fc-engineered version of Omalizumab harboring a mutation in the Fc portion to reduce binding to FcγRs. We then demonstrate that this mAb is equally potent as Omalizumab at blocking IgE-mediated allergic reactions, but does not induce FcgR-dependent adverse reactions. Therefore, this Fc-engineered anti-IgE mAb might represent safer therapeutic solution for the treatment of allergic diseases.

Abstract No. P4

In vivo pathogenicity of IgG from patients with anti-SRP or anti-HMGCR autoantibodies in immune-mediated necrotising myopathy

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Objectives In autoimmunity, autoantibodies (aAb) may be simple biomarkers of disease or true pathogenic effectors. Immune-mediated necrotizing myopathies (IMNM) have recently been individualized among idiopathic inflammatory myopathies. They can be associated with anti-Signal Recognition Particle (SRP) and anti-3-Hydroxy-3-MethylGlutaryl-CoA Reductase (HMGCR) autoantibodies (aAb). IMNM are characterized by myofiber necrosis, mild inflammatory infiltrates and complement C5b-9 deposits at myofiber surface. *In vitro*, anti-SRP and anti-HMGCR aAb were recently shown to induce muscle fibre atrophy and impair myoblast fusion. In patients, the level of aAb correlates with IMNM activity and disease may respond to immunosuppression, suggesting that they are pathogenic. Thus, we aimed to evaluate the pathogenicity of IgG from patients with anti-SRP or anti-HMGCR aAb *in vivo* by developing the first mouse model of IMNM.

Methods IgG from patients suffering from anti-SRP or anti-HMGCR associated IMNM were passively transferred to wild-type, $Rag2^{-/-}$ or complement $C3^{-/-}$ mice. Immunocompetent mice were also immunized with recombinant SRP or HMGCR protein. Muscle deficiency was evaluated by grip test and muscle strength upon electrostimulation. Histological analyses were performed following haematoxylin/eosin staining, immunofluorescence or immunohistochemistry analysis. Antibody levels were quantified by addressable laser bead assay (ALBIA).

Results Passive transfer of anti-$HMGCR^{+}$ or anti-SRP^{+} IgG from patients suffering from IMNM to wild-type or $Rag2^{-/-}$ led to a significant decrease in muscle strength whereas IgG-depleted patients' serum or IgG from healthy individuals had no effect. Histologically, this was associated with myofiber necrosis accompanied by myophagocytosis by $F4/80^{+}$ macrophages and C5b-9 deposits. Pathogenicity of aAb was reduced in $C3^{-/-}$ mice while supplementation with human complement increased their pathogenic potency in wild-type mice. Breakage of tolerance by active immunisation with SRP or HMGCR also provoked disease.

Conclusion This is the first report to demonstrates that patient-derived anti-SRP^{+} and anti-$HMGCR^{+}$ IgG are pathogenic towards muscle *in vivo* in mice. This pathogenic effect is mediated in part through a complement-dependant mechanism, definitively establishing the autoimmune character of IMNM. These results support further treatment evaluation of plasma exchanges and complement-targeting therapies in IMNM.

Abstract No. P5

Characterization of Rituximab and C1q interactions on living cells in real-time

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One of the fastest growing drug classes are therapeutic antibodies and next-generation antibodies with improved properties are being developed. Knowledge of how antibody binding to its target epitope relates to function has the potential to aid in the design and selection of improved antibodies. As the cellular environment can influence the antibody-receptor interaction, characterizing the binding towards the intended, living target cell type, can give a better understanding of the *in vivo* situation compared to purified measurement systems. A method that was designed to study biomolecular interactions on living cells is LigandTracer. Being a real-time method, both the affinity and kinetic parameters of an interaction can be quantified. The interaction of the therapeutic anti-CD20 antibody Rituximab with Daudi cells was investigated in detail and found to display a heterogenous binding pattern [1]. To further characterize this interaction, kinetic analysis on living cells was combined with fluorescence quenching to study interaction properties in combination with antibody clustering on cells [2]. It was found that the Rituximab interaction can be modeled by assuming the presence of two interaction components that differ in binding stability. The affinity with which the Rituximab bound to the cells, changed depending on the antibody concentration. The more stable interaction component was dominant for lower concentrations, whereas the less stable interaction component dominated for higher concentrations. A likely explanation for this behavior is that the fraction of bivalently bound antibody molecules differs. To investigate if the concentration dependent binding behavior has any implications on functional aspects, a real-time binding assay with C1q to Rituximab opsonized cells is set-up, with the aim of providing a tool for studying how antibody binding relates to mode of action.

References:

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Abstract No. P6

Complement activation at the surface of HIV-1-infected cells

Jérémy Dufloo[1], *Julian Buchrieser*[2], *Florence Guivel-Benhassine*[2], *Valérie Lorin*[3], *Ludivine Grezlak*[2], *Guillaume Mestrallet*[2], *Emilie Dupouy*[2], *Katia Bourdic*[4], *Olivier Lambotte*[4], *Hugo Mouquet*[3], *Timothée Bruel*[5,*], and *Olivier Schwartz*[5]

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Anti-HIV-1 antibodies activate the complement system to opsonize and eliminate viral particles. However, the mechanisms regulating complement activation at the surface of infected cell are partly understood. Here, we show that only a subset of broadly neutralizing antibodies (bNAbs), targeting the CD4 binding site and the V3 loop, triggers C3 deposition and complement-dependent cytotoxicity (CDC) in cells engineered to express high surface levels of HIV-1 Env. Mutating the Fc region of bNAbs to enhance hexamerisation potentiates CDC. CD4+ T cells infected with various lab-adapted and primary HIV-1 strains and treated with bNAbs are susceptible to C3 deposition but not to CDC. The protection against CDC in primary T cells is mediated by CD59. Moreover, the viral proteins Vpu and Nef impair C3 deposition at the surface of infected cells, mediated either by bNAbs or by polyclonal IgGs from infected individuals. Altogether, our results highlight the importance of the complement for the antiviral activity of bNAbs and reveal a novel function of Vpu and Nef against this innate component of the immune system.

Abstract No. P7

Investigating the mechanisms of IVIg mediated resolution of autoimmune inflammation

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In the immune system immunoglobulins can play a dual role. First IgG has pro-inflammatory properties as the humoral component of the adaptive immune system, being important in fighting diseases by leading to acute and limited inflammation. But when it comes to a loss of tolerance it can also cause chronic inflammation by cytotoxic autoantibodies or autoantibodies in the form of immune complexes, activating pro-inflammatory effector pathways. Secondly and in contrast to the pro -inflammatory properties, polyclonal IgG preparations consisting of the pooled serum IgG from thousands of healthy donors administered in a high dose (intravenous immunoglobulin, short: IVIg) can resolve acute and chronic inflammation very efficiently in a broad variety of diseases. In the past years many research studies have focused on determining the mode of action underlying the IVIg-mediated resolution of inflammation. However, most of the existing knowledge in this research field comes exclusively from experiments carried out with a preventive IVIg treatment approach. Thus, the aim of our studies was to investigate the difference between IVIg's mode of action in the widely used preventive treatment, in comparison to a therapeutic IVIg treatment of already established inflammation in a murine model of inflammatory arthritis.

Our experiments show that under therapeutic treatment conditions IVIg resolves inflammation in at least two different pathways in inflammatory arthritis. One resolving acute neutrophil mediated joint inflammation depending on terminal sialic acid residues and the presence of the neonatal Fc Receptor on hematopoietic cells but independent of an intact splenic architecture, the cytokines IL4 and IL33 and further downstream STAT6. The second pathway seems to lead to the prevention of bone destruction by osteoclasts and is independent of IVIg sialylation but requires the inhibitory FcgRIIB. Our studies suggest that the pathways underlying preventive and therapeutic IVIg treatment of autoimmune diseases differ, suggesting that more work is necessary to understand IVIg activity under clinically relevant conditions.

Abstract No. P8

Assessment of B cell reactivity and clonality at the single-cell level

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The assessment of adaptive immune receptor repertoires (AIRR) at the single-cell level provides information typically not available in bulk sequencing approaches, such as chain linkage, cell-based error correction and a direct measurement of clonal expansion. In addition, some single-cell platforms facilitate the rapid recombinant expression of Igs/TCRs for reactivity assessment and the association of the analyzed cells with their flow cytometric phenotype. Such platforms have recently demonstrated their utility, e.g. by expanding our understanding of the affinity maturation against complex antigens like *Plasmodium* surface antigens.

However, single-cell methods also pose new challenges for experimental quality control and subsequent analysis. The high complexity of single-cell NGS library requires sensitive controls for chimeric amplicons and "adaptor hopping" related artifacts. Furthermore, the definition of clonal relation needs to be adapted for a situation in which information on multiple loci is often present but potentially incomplete due to random drop-outs during amplification. In this talk I will present an overview of these topics and as well as discuss our recent insights and possible solutions.

Abstract No. P9

Characterizing autoantibody-producing cells in thrombocytopenic patients for secretion rate and affinity for a platelet autoantigen

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Immune thrombocytopenia (ITP) is characterized by autoantibody-mediated destruction of platelets resulting in the occurrence of potentially life-threatening bleeding episodes. Current treatments include B cell depletion and total splenectomy with success rates for each of ~50%. Splenectomy is performed to eliminate both the site of platelet destruction and of autoantibody production by plasma cells, but patient responses vary from no effect to full recovery. A functional characterization and anatomical localization of autoantibody-producing cells in ITP patients appears crucial for the better understanding of this pathology.

Herein we used a droplet-microfluidic system that allows determining the rate of secretion and affinity of IgG antibodies for one of the target antigens on human platelets, GPIIbIIIa, in a single-cell manner for about 6,000 cells (20,000 droplets) per chip within 1 hour. To analyze the distribution of anti-GPIIbIIIa IgG-producing cells in different anatomical sites, we characterized cells from the spleen of 7 ITP patients that underwent splenectomy, the bone marrow aspirates of 5 patients, and PBMCs from some of the same individuals. Most cells secreted IgG at rates from 10-300 molecules per second, with the highest rates being found among bone marrow cells. The proportion of GPIIbIIIa-autoreactive cells and the affinity of the IgGs they produced was heterogenous amongst patients, but in most cases, we identified a proportion of cells producing antibodies with detectable affinity for GPIIbIIIa.

We continue including more ITP patients and accumulate single cell data on patients. The understanding of autoreactive plasma cell distribution and phenotype should provide ways to further categorize ITP patients and understand the underlying immune mechanism.

Abstract No. P10

Anti-IL-4 and anti-IL-13 dual vaccination using Kinoid technology prevents development of allergic airway inflammation in mice

Eva Conde[1, 2,*], *Romain Bertrand*[2], *Bianca Balbino*[1], *Jonathan Bonnefoy*[2], *Julien Stackowicz*[1], *Noémie Caillot*[2], *Fabien Colaone*[2], *Pierre Bruhns*[1], *Vincent Serra*[2], *Géraldine Grouard-Vogel*[2], and *Laurent Reber*[1, 3]

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Background: Allergies represent a major public health issue of increasing prevalence that, in most cases, lack efficient long-term therapy. Allergies are considered as a consequence of a tolerance breakdown, resulting in type 2 immune responses characterized by overproduction of cytokines such as interleukin 4 (IL-4) and IL-13. IL-4/IL-13 kinoids are conjugated vaccines consisting of mouse IL-4 and IL13 molecules chemically coupled to CRM_{197}, a non-toxic diphtheria toxin mutant, serving as a carrier protein. Immunizations with these kinoids elicit the production of polyclonal anti-IL-4/IL-13 neutralizing antibodies.

Aim: Investigate if vaccination with IL-4/IL-13 kinoids would be beneficial in a mouse model of allergic asthma.

Methods: Allergic asthma was induced in BALB/c mice by repeated (biweekly) intranasal administrations of house dust mite (HDM) for six weeks; this model recapitulates the main clinical hallmarks of asthma such as chronic airway inflammation and structural airway remodeling. To assess the effect of kinoid vaccination, mice received four intramuscular injections of IL-4 kinoid (IL-4-K), IL-13 kinoid (IL-13-K) or a combination of both (combo-K), all in a squalene-based adjuvant emulsion. Respiratory functions were evaluated by whole-body or invasive plethysmography, airway and lung inflammation by histology or flow cytometry, antibody production by ELISA, and antibody neutralizing capacities by cellular bioassays.

Results: High titers of anti-IL-4 and/or IL-13 neutralizing antibodies were detected in sera from kinoid-immunized mice. HDM-induced airway hyperreactivity, airway and lung eosinophilia and IgE levels were partially reduced in mice vaccinated with either IL-4-K or IL-13-K. Combo-K vaccination, however, protected mice from this HDM-induced allergic asthma model.

Conclusion: Our results demonstrate that kinoids are capable to break B cell tolerance against IL-4 and IL-13, and protect from allergic airway inflammation induced by chronic HDM exposure in mice. The dual vaccination against IL4 and IL-13 therefore represents a promising new therapeutic strategy in the treatment of allergic inflammation and asthma.

Abstract No. P11

IgG hexamerization affects binding of Staphylococcal protein A: impact on complement activation

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Staphylococcal protein A (SpA) is a conserved virulence factor of Staphylococcus aureus (S. aureus), which is anchored to the bacterial cell wall and also secreted to the extracellular environment. SpA is composed of five homologous domains that bind to the Fc portion of immunoglobulins (Igs). This feature enables the bacterium to recruit Igs in the incorrect orientation, preventing their recognition by Fc-receptors of neutrophils. It was also shown that SpA is able to impair the binding of C1 to IgG1, although the mechanism by which the binding is affected remains to be clarified. Recent work showed that the classical complement pathway is more efficiently activated when target-bound IgGs form organized hexamers on the surface of tumor cells. As IgG hexamerization on cell surface occurs via nonconvalent Fc-Fc interactions between IgGs, we investigated whether SpA interferes with antibody hexamerization and consequently with C1 recruitment. We produced human monoclonal IgGs against DNP, both wild type and variants that harbor mutations to potentiate hexamer formation after antigen encounter (enhanced IgG variants). Using streptavidin-coated magnetic beads loaded with biotin-DNP, we measured by flow cytometry the binding of recombinant B domain of protein A (SpA-B) and purified C1 to different variants of anti-DNP IgGs bound to the beads. We confirmed that SpA-B is able to bind IgGs of different subclasses (IgG1, IgG2 and IgG4) on surface of DNP-coated beads and that the binding of SpA-B interferes with C1 deposition on IgG1 and IgG2. Interestingly, we have also verified that SpA-B binding impacts C1 deposition when C1 is already bound to IgGs on the bead surface. Additionally, we detected less binding of SpA-B to the enhanced IgG variants, which resulted in an increase of C1 deposition when comparing with the effect observed on wild type IgG molecules. Taken together, our studies provide a possible explanation for the impact of SpA on C1 deposition and encourage the study of enhanced IgG variants as a therapeutic strategy to clear S. aureus infections.

Abstract No. P12

Importance of functional antibodies in protection of pregnant women from malaria.

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Background: Malaria in pregnancy was responsible for 10,000 maternal deaths and 100,000 still births in 2018. Pregnant women, despite lifetime malaria exposure, are more susceptible to *Plasmodium falciparum* malaria infections than their non-pregnant counterparts, due to the ability of parasites to sequester in the placentaby expressing VAR2CSA, an erythrocyte membrane protein-1 variant antigen, which consists of six Duffy-like binding domains(DBL).Recent studies have shown an association between the presence of naturally-acquired antibodies (Abs) to DBL2 of the VAR2CSA protein, and reduced adhesion of infected erythrocytes to the placenta. The two leading placental malaria vaccine candidates PAMVAC and PRIMVAC both include the DBL2 domain. However, the capacity of Abs to DBL2 to trigger downstream effector functions such as Ab-dependent opsonophagocytosis and complement activation via their Fc regionsis poorly understood.

Methods: Customised bead-based multiplex assays were developed to identify key VAR2CSA DBL domain targets of Ab immunity in non-placental malaria-infected women (*N*=27) compared to women with placental malaria (*N*=50). The VAR2CSA screening included detectors for Abs (total IgG, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM) and Ab ability to engage with Fc receptors (FcγRI, FcγRIIa, FcγRIIIa, FcγRIIIb) and complement (C1q), resulting in the assessment of over 150 Ab features per subject. Multi-dimensional machine learning analysis including feature selection and partial least squares discriminant analysis,was used to identify key protective immune components.

Results: Multi-dimensional clustering analysis identified IgG (mainly IgG1 and IgG3) and C1q to be the major features associated with non-placental malaria.Confirming this, elevated levels of DBL2-binding IgG1 ($p=0.0023$), IgG3 ($p=0.0013$) and C1q (complement) activating Abs ($p= 0.0016$) were detected in non-placental malaria-infected women. DBL2-binding IgG1 and IgG3 also correlated with enhanced engagement with all investigated FcγRs (IgG1: $r = 0.673$-0.863; IgG3: $r = 0.665$-0.759, all $p<0.0001$) and C1q activation of the complement pathway (IgG1: $r = 0.7176$, $p <0.0001$; IgG3: $r = 0.799$, $p<0.0001$).

Conclusion: Our studies show that non-placental malaria is distinguished by IgG1 and IgG3 Abs that bind to DBL2 of VAR2CSA and we suggest this association with protection from placental malaria is potentially mediated through the activation ofcomplement and Fc receptor functions.Importantly, thetwo leading placental malaria vaccine candidates include recombinant DBL2 proteins. Understanding the Ab mediated activation ofcomplementand Fc receptor functions could provide vital information to guide the rational development of these vaccines.

Abstract No. P13

Immune complexes activate neutrophils in human anaphylaxis

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Anaphylaxis is a systemic allergic reaction that can lead to multiple organ failure and death. Its classical mechanism involves histamine release by basophils and mast cells after cross-linking of FceRI-bound specific IgE by allergen. However, models of anaphylaxis in mice demonstrated the existence of another pathway induced by IgG immune complexes (IC). Using active and passive anaphylaxis mouse models, we previously demonstrated that neutrophils play a key role in IgG anaphylaxis. We thus investigated neutrophil activation by IC *in vitro* and *in vivo* in human anaphylaxis by the mean of a multicentric translational study.

Tri-nitro phenyl (TNP)/anti-TNP ICs were prepared using chimeric anti-TNP antibodies of different human isotypes (IgG1-4 and IgA). With these IC we stimulated whole blood or purified neutrophils and analyzed their activation by flow cytometry. In parallel, 86 patients with a strong suspicion of perioperative anaphylaxis and paired controls were included in the clinical study. Blood was collected within 30 minutes after anaphylaxis onset and during allergological evaluation 8 weeks later (baseline). Concentrations of elastase and neutrophil extracellular traps (NETs), and activity of PAF-acetyldhydrolase were measured in the plasma and neutrophil activation markers were monitored on fresh blood.

Stimulation of whole blood or isolated neutrophils by IgG IC resulted in CD11b upregulation, CD62L shedding, downregulation of CD16 and CD32a, degranulation, and netosis. Intriguingly, IgA and all IgG subtypes induced the same level of neutrophil activation. The activation was dependent of FcgRIIa and FcgRIIIb for IgG ICs and FcaRI for IgA ICs. In anaphylaxis patients, we found higher levels of NETs, elastase, CD11b and CD66b as compared to baseline and healthy controls. The activity of PAF-AH was lower in patients as compared to controls. All these features were linked to anaphylaxis severity.

We demonstrate that human immune complexes of all IgG isotypes activate human neutrophils, which undergo degranulation and netosis. In the context of perioperative anaphylaxis, patients showed high levels of neutrophil activation markers, which correlated with anaphylaxis severity. We thus propose that the IgG pathway is relevant in human anaphylaxis and that investigation of this new mechanism could lead to new diagnostic and therapeutic approaches.

Abstract No. P14

Polymorphic variant of IgG2 is less efficient in complement activation

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The humoral immune response to pathogens is shaped by the isotype, subclass and glycosylation profile of the antibodies produced by B-cells. Polymorphisms in the immunoglobulin gamma heavy chain gene form yet another layer of variation to the humoral antibody response which has never been studied in detail.

We produced all 27 known IgG allotypes with anti-TNP hapten specificity and assessed complement deposition with ELISA and complement dependent cytotoxicity (CDC) of TNPlated red blood cells (RBC) or Raji cells. CDC of TNPlated RBCs was most efficient with the IgG3 subclass, followed by IgG1, IgG2 and IgG4 respectively. However, in the CDC experiments where TNPlated Raji cells were used as target cells, IgG1 was superior in inducing CDC (IgG1>IgG3>IgG2>IgG4), suggesting that the target cell differentially influences IgG1/IgG3 mediated CDC. While only minor differences were observed within subclass for IgG1, IgG3 and IgG4 allotypes, allotype-specific variation was found for IgG2. Allelic variant IGHG2*06 showed less efficient complement activation and CDC activity compared to other IgG2 variants. This difference was more pronounced at the level of C3 deposition and CDC, than at the level of C1q binding. IgG2 allotype *06 expresses a unique serine at position 378 in the CH3 domain. Introducing this amino acid in an IgG1 background (A378S) reduced CDC efficiency, suggesting that the variation at position 378 interferes with complement activation independent of IgG subclass. Residue 378 is located near the CH2:CH3 elbow and might be involved in the IgG Fc interactions necessary for hexamer formation. IgG2 allotype *06 is frequently (prevalence of 20-40%) observed in African countries and tribes, encouraging further research on susceptibility to bacterial infections, in particular situations in which complement activation via IgG2 is involved in pathogen clearance.

Together these results point towards a variation in the capacity of IgG allotypes to activate complement, which might give new insights on susceptibility to infectious-, allo- or auto-immune diseases.

Abstract No. P15

Statistical design for effector function engineering of hexameric Fc domains

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Currently autoantibody-mediated diseases are treated with intravenous immunoglobulin (IVIG). By blockade of Fc gamma receptors, IVIG is thought to inhibit autoantibody effector functions and subsequent pathology. We aimed to develop recombinant molecules with enhanced Fc receptor avidity and thus increased potency over IVIG. Here we describe the molecular engineering of human Fc hexamers and explore their therapeutic and safety profiles. We show Fc hexamers were more potent than IVIG in phagocytosis blockade and disease models. However, in human whole-blood safety assays incubation with IgG1 isotype Fc hexamers resulted in cytokine release, platelet and complement activation, whereas the IgG4 version did not. We used a statistically designed mutagenesis approach to identify the key Fc residues involved in these processes. Cytokine release was found to be dependent on neutrophil FcγRIIIb interactions with L234 and A327 in the Fc. Therefore, Fc hexamers provide unique insights into Fc receptor biology.

Abstract No. P16

Eccentric associations: interaction of antibodies with cofactors

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By specific binding to proteins, carbohydrates and lipids, antibodies play an essential role in the defence of organisms against pathogens. In addition to the antibodies recognizing complex antigens, normal human immune repertoires contain a considerable fraction of antibodies that interact with high affinity to various low molecular weight organic molecules. Importantly, these molecules include some essential biological cofactors and prosthetic groups such as heme, ATP, FAD as well as some metal ions. Despite identification of cofactor-binding antibodies as a normal constituent of the immune repertoires, their fundamental characteristics have not been systematically investigated. Our previous studies demonstrated that the binding of heme to some antibodies results in an acquisition of novel antigen-binding specificities. Thus, the cofactor binding might represent an unconventional strategy for diversification of immune repertoire. Here we address three fundamental questions about the antibodies interacting with cofactor molecules. First, we describe our results from studies of the prevalence and immunological origin of cofactor binding antibodies in normal human and murine immune repertoires. Next we discuss our data from studies on molecular mechanisms of cofactor-mediated diversification of antibody binding specificity. Finally we present data emphasizing the pathophysiological functions of cofactor-binding antibodies and their potential to be exploited as a new type of therapeutics.

Abstract No. P17

Bacterial killing by complement requires immediate insertion of MAC precursor C5b-7

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An important effector function of the complement system is to directly kill Gram-negative bacteria via Membrane Attack Complex (MAC) pores. MAC assembly is initiated when C5 convertases cleave C5 into C5b, which together with C6, C7, C8 and multiple copies of C9 form the MAC pore (C5b6-9). Recently, we found that the formation of bactericidal MAC pores requires local conversion of C5 by C5 convertases at the bacterial surface. In this study we aimed to gain more insight into why local assembly of MAC pores is essential for bacterial killing. Here we show that rapid binding of C7 to C5b6 drastically enhances its capacity to form MAC pores that can damage both the outer and inner membrane. Binding experiments with fluorescently labeled C6 show that C7 prevents release of C5b6 from the bacterial surface. While released C5b6 can still permeate the bacterial outer membrane, it is incapable of damaging the inner membrane which is essential for bacterial killing. Trypsin shaving experiments and atomic force microscopy revealed that immediate binding of C7 to C5b6 is needed to stably insert C5b67 complexes into the bacterial membrane. Finally, using complement-resistant *E. coli* strains we show that bacterial pathogens can prevent complement-dependent killing by specifically blocking stable insertion of C5b67. While C5 convertase assembly is unaffected, these resistant strains do not allow stable insertion of C5b67 and MAC. Altogether, these findings provide basic insights into how bactericidal MAC pores are assembled in a target-specific manner.

Abstract No. P18

Evaluation of Antibody Response to Prokaryotic and Plant-Made Recombinant Leishmaniasis Antigens

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Visceral leishmaniasis (LV) is a serious zoonotic disease that can lead to death. The disease is a serious health problem, being endemic in more than 80 countries and its prevalence exceeds 1 million cases worldwide. This study aimed to develop effective recombinant vaccine antigen, conferring protection against *Leishmania chagasi*, as well as the development of a diagnostic kit for canine visceral leishmaniasis. The sequence encoding K39 (K39 antigen of the *L. chagasi* kinase region) was successfully synthesized and optimized for expression in the eukaryotic (plant) and prokaryotic systems. For bacterial production, the synthesized gene was subcloned into expression vector pET28a and expressed in *Escherichia coli* strain BL21 (DE3). In the transient expression in *Nicotiana benthamiana* the K39 gene was cloned into expression vector pCAMGATE-Hydrofobin. Different groups of BALB/c mice were immunized with the *E. coli* k39 protein, either in the purified form (rk39), or with the inactivated bacterium containing the expression plasmid and the protein (BL21-rK39), after immunization the profile immunological response was evaluated. The groups immunized with the k39 protein (rK39 and BL21-rK39) showed high production of specific antibodies already in the primary response with high levels of IgG and subclasses IgG1 and IgG2a, when compared to the group that did not present the protein in its composition (BL21- Mock). These groups also had high levels of IL-4 in the liver. Plant-based and diagnostic-directed (ELISA) rK39, against sera from dogs positive for visceral leishmaniasis, presented a sensitivity of 90.7% and specificity of 97.5%. The eukaryotic platform is a viable and promising option in the production of antigens for use in the immunodiagnosis of dogs with leishmaniasis, as well as in the production of antigens directed to immunization of animals. The k39 protein showed ability to activate the immune response in mice used as an animal model, not only for diagnostic use but also for its potential to be used as an immunogen.

Abstract No. P19

Contribution of human immunoglobulin G subclasses to antibody-mediated reactions in a fully FcγR-humanized mouse model.

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Immunoglobulin G (IgG) is the most abundant immunoglobulin in human serum and can be divided into 4 distinct subclasses. Each subclass contains a unique constant region that determines the antibody capacity to bind to IgG receptors (FcgR) or complement, and thus their biological functions. In order to assess the potential of the different IgG subclasses to trigger Fc-dependent reactions, we generated anti-TNP (Trinitrophenyl-) chimeric antibodies carrying the same paratope. We also produced Fc engineered variants of human IgG1 (hIgG1), already known to reduce FcgR and/or complement binding.

We tested the capacity of the different hIgG variants to trigger Fc-dependent passive systemic anaphylaxis in a mouse model, in which all endogenous FcgR were replaced by the complete human FcgR family ($hFcgR^{KI}$) or in mice that lack all FcgR ($FcgR^{null}$). Anaphylaxis was induced by intravenous transfer of anti-TNP hIgG followed by injection of TNP-coupled Bovine Serum Albumine (TNP-BSA) 16 hours later. Changes in body temperature were recorded over time. In order to evaluate the complement binding capacity of the different IgG variants, we developed an in vitro assay, for which hC1q binding to antibodies bound on TNP-BSA coated plates was revealed in a sandwich ELISA assay.

Our results demonstrate that monoclonal human IgG can trigger anaphylactic reactions in $hFcgR^{KI}$ mice upon challenge with their specific antigen. Human IgG1, hIgG2 and hIgG3 were capable of anaphylaxis induction, whereas hIgG4 did not trigger this reaction. Insertion of mutations in the Fc region aimed to reduce hIgG1 binding to FcgR, “LALA” or “NA”, abrogated the anaphylactic reaction, while mutations targeting complement binding sites in the Fc portion, “KA”, had no effect. $FcgR^{null}$ mice did not undergo anaphylaxis induced by any of the IgG variants tested, this is surprising as several human IgGs notably IgG1>IgG2, IgG3 and IgG1-NA bound C1q in our in vitro assay. This suggests that although these IgG can trigger the classical complement pathway, that its strength is insufficient in this model to yield symptoms of anaphylaxis. No C1q binding was detected with IgG4, IgG1-LALA nor -KA antibodies.

In conclusion, we show that hIgG1-3 all engage FcgR and C1q, with hIgG1 being the most potent subclass to do so. Human IgG4 is incapable of triggering anaphylaxis in $hFcgR^{KI}$ mice and also does not bind C1q, suggesting that it could antagonize effector functions mediated by other subclasses.

Abstract No. P20

Squaring in vitro complement assays with in vivo results: case studies in anti-HIV-1 complement activity

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The role of the complement system in HIV-1 immunity and pathogenesis is multifaceted, and better understanding of anti-HIV-1 antibody-mediated complement activities would inform prophylactic and therapeutic development efforts. Unfortunately, the multitude of factors influencing complement regulation and activation *in vivo* makes *in vitro* recapitulation difficult.Here, we present case studies demonstrating how different *in vitro* measures relate to one another, how conflicting biophysical and functional results might be reconciled, and implications on the interpretation of past and design of future studies.

To assess the functional impact of antibody mediated complement activity, a panel of C1q-knockout and -enhancing IgG1 Fc amino acid sequence variants were constructed for a set of HIV-specific antibodies targeting distinct epitopes. This antibody panel was evaluated for antigen binding, and C1q binding and functional activity in an antigen-independent manner to verify that reported phenotypes were transferrable. Other assays, including complement-dependent cytolysis, conducted in an antigen-dependent context yielded some results inconsistent with biophysical phenotypes, suggesting a high degree of epitope and context dependence, consistent with the highly avid engagement by C1q associated with efficient classical activation. Collectively, this *in vitro*testing suggests that the degree of antibody-mediated complement activity, and thus the role complement plays in antibody-mediated HIV-1 protection, may not be generalizable, but instead must be considered on an antibody-by-antibody, case-by-case basis. These observations also motivate reinterpretation of the potential role of complement in the anti-viral activity of antibodies to HIV defined by previous passive transfer and viral challenge experiments in animal models.

Moving forward, we intend to leverage the insights gleaned from thesestudies to inform nonhuman primate passive immunization studies better suited to address the role of complement in antibody-mediated protection against HIV-1 infection. The outcome of such studies will enable assessment of the relative importance of complement-mediated functions, if any, in the protection against HIV-1 acquisition, which may help inform vaccine design and prophylactic antibody development efforts.

Abstract No. P21

Generation of monoclonal antibodies specific for microorganisms in drinking water

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Introduction:

Contamination of drinking water by pathogenic microorganisms (pMOs) is a great concern worldwide and leads to several infections in humans. The detection of these pMOs is performed mainly by sampling bacterial cultures which is dependent on cultivation and reproduction times. Alternative used methods such as flow cytometry (FC) or molecular analyses are developed to improve the monitoring of water quality. In terms of antibody-based detection methods the generation of specific mABs represents a particular challenge due to the complexity of pMOs as antigenic target.

Methods:

In this study we combined comparative genomic analysis,*in silico*epitope prediction, novel immunization strategies and hybridoma cell sorting methods to generate specific monoclonal antibodies against*E.coli*. A comparative genomic analysis based on next generation sequencing data of 50 microbial genomes was performed to identify*E.coli*specific antigens useful for immunization. The genes were analyzed*in silico*for surface expression and antigenicity. Based on these results an epitope predicition was perfomed and the most promising epitopes were cloned into viral coat proteins (VPs). After immunization the B lymphocytes of mice were fused with our recently developed transgenic myeloma cell line. The fused hybridoma cells were modified with an antibody capture matrix which enables the direct linkage between the secreted antibody and the producing hybridoma cell. An optimized labeling-protocol with pMOs as antigen was established to sort specific hybridoma cells producing high affinity antibodies against*E.coli*.

Results:

From 226.735 genes we obtained a total of 10 genes which encode potential cell surface protein candidates. The resulting proteins were analyzed for linear B cell epitopes which are accessible and antigenic. The epitopes with the highest scores were cloned into viral coat proteins used for immunization. Five of six immunized mice showed a specific immune response against*E.coli.*After performing our labeling procedure we sorted the hybridoma cells with the highest signals to obtain antibody producing cells with a high affinity against*E.coli*. From three cell-fusions we received 13.700 to 56.000specific cells per fusion. Although not all cells survived the labeling and sorting procedure 120 monoclones along with over 300 polyclones per fusion have been screened by FC. The resulting data showed a high sorting efficiency up to 90 % of positive antibody producing cells against*E.coli*.

The process shown here is therefore a suitable approach to generate specific mAbs against complex targets as pMCs without the need of using whole pMCs for immunization.

Abstract No. P22

Post B-cell sialylation in mice analyzed by xCGE-LIF

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Immunoglobulin G (IgG) is one of the key molecules in humoral immunity. Its effector functions are engaged through interactions with various Fcγ and other receptors and depend (among other things) on the type of the N-glycan structure attached to the Fc portion of the molecule. The presence of terminal sialic acids on this glycan is considered responsible for the anti-inflammatory properties of IgG. Recently it was proposed that IgG sialylation in mice can occur in the extracellular environment of the bloodstream, independent of the B-cell secretory pathway. Trying to verify and further investigate this finding, the aim of this study was to confirm the existence and examine the extent of the B-cell independent sialylation of IgG in mice. To this purpose, desialylated intravenous immunoglobulin (IVIg) preparation was administered intravenously to ST6Gal1$^{-/-}$ and μMT$^{-/-}$ mice (lacking endogenous IgG) on C57BL/6 background. Blood was collected before and 2, 4 and 6 days after IVIg administration, IgG isolated from the serum on a protein G monolithic plate, glycans released by protein N-glycosidase F, labeled with 8-aminopyrene-(1,3,6)-trisulfonic acid and analyzed by multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF). Data analysis was performed by glyXtool software. We observed no change in the N-glycan profile of desialylated IVIg in the ST6Gal$^{-/-}$ mice. However, new glycan structures corresponding to mouse IgG sialylated N-glycans were observed in μMT$^{-/-}$ mice at low levels, indicating a presence of extrinsic sialylation in the mouse bloodstream.

Abstract No. P23

Disruption of Gram-negative bacteria by human serum requires complement and lysozyme.

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The human immune system employs humoral and cellular mechanisms to kill invading Gram-negative bacteria. The bactericidal activity of human serum largely depends on the action of the complement system, a family of proteins that generates bactericidal membrane attack complex (MAC) pores. Additional protection against Gram-negative bacteria is provided by human phagocytes that can engulf bacteria and destroy them intracellularly. Apart from MAC and phagocytes, the role of other human antimicrobial proteins and peptides in defense against Gram-negatives is less well-understood. This is mainly due to the fact that these factors cannot penetrate the impermeable Gram-negative outer membrane that physically surrounds the inner membrane and peptidoglycan layer. Recently we showed that killing of Gram-negative bacteria in human serum depends on the MAC that efficiently kills bacterial cells by damaging both the outer and inner membrane. By generating MAC pores in a semi-purified manner, we also noticed that MAC-induced cell death does not affect the intactness of the bacterial cell particle. Here we show that human serum effectively damages Gram-negative bacteria in such a way that it triggers shape loss and disruption of bacterial particles. Serum-induced disruption of bacteria depends on the combined action of the MAC and lysozyme, a muramidase that degrades peptidoglycan. Our data suggest that MAC-mediated outer membrane damage allows lysozyme to disrupt the underlying peptidoglycan layer. A combination of flow cytometry and confocal microscopy experiments suggest that the MAC also enhances the intracellular disruption of Gram-negative bacteria after phagocytosis. Altogether, these data provide insight into how different arms of the immune system work closely together to efficiently clear invading Gram-negative bacteria.

Abstract No. P24

On target hexamerisation of IgG1 driven by a mutant form of IgM tail-piece C-terminal fusion

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The majority of monoclonal antibodies (mAbs) approved for use elicit a therapeutic response via Fc-mediated mechanisms, such as the recruitment of C1q to enable complement-dependent cytotoxicity (CDC). C1q recruitment has been shown to be optimally activated when antibody Fc molecules are arranged hexamerically at the target cell surface. Fusion of the C-terminal 'tail-piece' sequence of IgM promotes the hexamerisation of human IgG and Fc domains. The tail-piece contains a penultimate cysteine residue which is known to be critical for hexamer formation and stabilisation. Mutation of this cysteine to serine ablates hexamer formation from mammalian cell expression. We show that monomeric Fc or IgG containing such C575S mutants retain the potential to form non-covalent, reversible hexamers. Fc-tailpiece (C575S) form hexamers after concentration. IgG1-tailpiece (C575S) have enhanced ability to kill target bearing cells by complement mediated events – consistent with 'on target' hexamer formation. Hence C575S mutant IgM tail-pieces represent an alternative mechanism for augmenting CDC activity of IgG or promoting on-target antibody multimerisation.

Abstract No. P25

A novel mouse model for human melanoma: new possibilities to test immunomodulatory antibodies in the context of a human immune system

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The finding that immunomodulatory monoclonal antibodies can enhance human immune responses has revolutionized the field of cancer research in recent years. However, most of the existing knowledge in this research field comes from studies carried out in murine mouse model systems or in vitro cultures, in which the environment of the tumor differs highly from the one found in the human system, potentially leading to results that cannot be fully translated into human cancer therapy. Therefore, it is crucial to generate new mouse models, which allow the study of cancer immunotherapies in the context of a human immune system. We focused on the establishment of a human melanoma-like tumor in a humanized mice model, thereby being able to analyze different parameters, such as tumor infiltrating human cells and cytokine production. The subcutaneous injection of human melanoma cells led to the growth of solid tumors, which where infiltrated by both human and mouse cells. Interestingly, the intravenous injection of human melanoma cells resulted in a massive expansion of human T-cells and detectable tumors in liver, lung and kidney.

Furthermore, our humanized mouse model enables us to study novel cancer immunotherapies in the context of a human immune system. The particular feature of this model is that all immune cells lack functional mouse activating Fc**γ**-receptors, enabling us to solely study the role of human Fc-Fc**γ**-interactions for immunomodulatory antibodies. We focused on understanding how the immunomodulatory agonistic antibody a-CD137 works in the context of a human immune system and how its in vivo activity can be modulated by its Fc-part. We show that the injection of a-CD137 into humanized mice leads to a massive proliferation of mainly CD8+, CD4+CD8+ and CD4-CD8- T cells in the blood and organs of humanized mice. Moreover, treatment with the deglycosylated a-CD137 antibody showed a delayed T cell proliferation, suggesting a dependence on Fc**γ**R. Furthermore we could observe accumulation of liver T cells upon antibody injection. These findings show the potential of our humanized mouse model to conduct preclinical studies with therapeutic antibodies and specially focus on the role of Fc-Fc**γ**R interactions, the side effects of the antibodies of interest and on their anti-tumor activity.

Abstract No. P26

Extracellular ATP can mediate cleavage of CD27 to generate CD27-IgD- B cells in circulation of hantavirus patients.

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Hantaviruses are rodent-borne viruses causing potential lethal infections in humans. Different hantaviruses exist worldwide, reporting a fatality rate of up to 40%. The Puumala hantavirus, endemic in northern Sweden, has a relatively low fatality rate but the hospitalization rate is high. No vaccine to the virus and no treatment for the disease exist. Despite differences in severity, the immune-mediated pathogenesis of Puumala virus infection is similar to that of highly lethal strains of hantavirus. Puumala infections cause hemorrhagic fever with renal syndrome (HFRS), major symptoms of the disease being thrombocytopenia and transient kidney dysfunction. We set out to characterize how the humoral immune response is affected during infection and if these two major symptoms have a different effect on this.

Here, we demonstrated that thrombocytopenia and transient kidney dysfunction differentially affect the humoral immune response. Whereas low thrombocyte counts were strongly associated with an abnormal high frequency of plasmablasts (PBs) in circulation, kidney dysfunction was associated with accumulation of $CD27^{-}IgD^{-}$ B cells and $CD27^{low/-}$ PBs in circulation. Although the frequency of PBs correlated with thrombocytopenia, we identified that increased serum creatinine levels, used as proxy for kidney dysfunction, associated with an increase in longitudinal neutralizing activity of plasma antibodies in HFRS patients. The $CD27^{-}IgD^{-}$ B cells identified in the patients with increased kidney dysfunction comprised a heterogenous population of activated and resting cells, where on average approximately 50% were $Fcrl5^{+}$ atypical B cells. Furthermore we identified that high levels of extracellular ATP in plasma during acute HFRS contributed to downregulation of surface CD27 on B cells, and that this was affected by the activity of matrix metalloproteinase 8.

Collectively, we here demonstrate an association between reduced kidney function, longitudinal development of neutralizing antibodies and accumulation of atypical B cells in circulation during HFRS. This implies that a high frequency of atypical B cells is not detrimental, but might actually be beneficial for long lasting humoral immunity. Also we show that increased levels of extracellular ATP during infection could be responsible for an increased frequency of $CD27^{low/-}$ B cells.

Abstract No. P27

An in vitro system to study diversification of antibodies by large DNA insertions

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A key feature of humoral immunity is the ability to recognize a wide range of antigens by a vast number of antibodies secreted by B cells. Diversity of the antibody genes is generated by somatic recombination and hypermutation of its so called variable region. Recently we described that non-immunoglobulin sequences deriving from distant genomic regions can integrate into the antibody gene locus. Moreover, we have evidence that some inserted exon-sequences can be successfully spliced into immunoglobulin transcripts or directly integrate into the variable region preserving its reading frame. Although rare, the presence of various in frame insertions in antigen-experienced cell populations suggests that non-immunoglobulin inserts may contribute to the diversity of antigen recognition sites. Although insertions were detected in the heavy chain genes of memory B cells isolated from blood, it is unclear if they are acquired after*in vitro*stimulation. To this end, we activate naïve B cells by various combinations of B cell receptor, Toll like receptor and cytokine stimuli. Our data show that *in vitro*activation of class switch recombination is accompanied by DNA insertions in antibody transcripts suggesting that AID-induced double strand breaks may serve as integration sites. To understand if insertions may also derive independently from class switching or AID activation, we are currently setting up screening systems to detect DNA-insertions in light chains of antibodies and T cell receptors, respectively. To gain further mechanistic insights, the established *in vitro*system can be manipulated to study the molecular players involved in the diversification of antibodies by large DNA insertions.

Abstract No. P28

Tracking Temporal and Spatial Affinity Maturation of Antibodies from Single-Cells in a mouse model of autoimmunity

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The emergence and dynamics of antibody secretion in plasma cells (PCs) responsible for the outbreak of autoimmunity in a clinically-relevant mouse model of rheumatoid arthritis are difficult to study. The K/BxN model is derived from mice who express a transgenic TCR that recognizes an epitope of the ubiquitously expressed glucose-6 phosphate isomerase (GPI) when presented on the MHC A^{g7} molecules on B cells from a NOD background. GPI naturally deposits on articular cartilage, and the presence of anti-GPI IgG autoantibodies produced in high levels in this model induces immune complex formation, joint inflammation, and arthritis-like symptoms manifesting at an age of 3-6 weeks. Tracking the temporal and spatial niching of these autoantibody-secreting PCs and their affinity maturation may reveal insights into the causes of autoimmune outbreaks. Since antibody-secreting PCs lack surface expression of IgG, surface-labeling techniques are inadequate to analyze the emergence and secretion rates of these autoantibodies.

To overcome this, we developed a droplet microfluidics-based platform integrated with kinetic image acquisition that encapsulates single cells with a fluorescent sandwich ELISA into picoliter droplets. Rates of secretion and affinity of the IgGs against GPI are estimated for hundreds of single PCs simultaneously. Single PCs from bone marrow (BM), spleen (SP), and lymph nodes (LNs) were encapsulated from K/BxN mice at various stage of the disease, from early onset to late stages. Estimated affinities for the secreted antibodies against GPI suggest that the evolution of affinity and kinetics differs between the LNs, BM, and SP.

Our results suggest that the maturation of anti-GPI IgGs secreted by single PCs evolve heterogeneously in different organs during an autoimmune outbreak in the K/BxN model. Through this work, we can begin to create anatomical maps to track the affinity maturation and antibody secretion rates for autoimmune diseases.

Abstract No. P29

Antibody diversification through non-VDJ insertions

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Antibody diversity is crucial for the vertebrate immune system and is formed by the somatic recombination and imprecise joining of the variable (V), diversity (D) and joining (J) segments. Recently, a new mechanism of antibody diversification was discovered, whereby a non-VDJ-sequence is inserted into the antibody heavy chain gene and becomes the pivotal antigen-binding element. Conventional repertoire studies do not allow efficient detection of such hybrid antibodies due to methodological limitations. To address this problem, we designed an approach based on suppression PCR to selectively amplify and study rare, extra-long antibody mRNA templates. Analysis of 48 healthy individuals showed that in every donor studied immunoglobulin transcripts contained inframe non-VDJ-insertions in 1 out of 10^5 peripheral blood B cells. Insertions were positioned either in the variable joint-region between V and DJ or VD and J, or they were located downstream of an entire VDJ and upstream of the constant region (VDJ-C insertions). Interestingly, we discovered that VDJ-C insertions originate primarily from telomere proximal regions and the majority of them consists of complete exons. The latter suggests that the insert is incorporated in genomic DNA and consecutively spliced into the antibody transcript. By contrast, more than two-thirds of V-D-J insertions are represented by introns and intergenic sequences. One-sixth of all hybrid transcripts detected contain mitochondrial DNA and are exclusively inserted inside the VDJ. These results suggest separate mechanisms by which V-D-J and VDJ-C insertions emerge. Detection of hybrid antibodies in switched memory B cells indicates that this novel mechanism can contribute to antibody diversity and suppression PCR is a robust and sensitive method to screen for these novel antibodies.

Abstract No. P30

Antigen targeting of Fc receptors induces strong and functional relevant T cell responses in vivo independent of ITAM signaling but dependent on dendritic cell subsets

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Dendritic cells (DCs) are important antigen presenting cells (APCs) and induce immune responses, but also preserve peripheral tolerance. We showed the preferential induction of either $CD4^+$ or $CD8^+$ T cell responses by DC subpopulations *in vivo* by targeting antigens to endocytic C-type lectin receptors. The also highly endocytic active Fc receptors (FcRs) enable APCs to take up antigens in form of immune complexes. As they are expressed on various APCs, we aimed to identify responsible APCs for primary and secondary immune responses by using our antigen delivery by recombinant antibodies to activating and inhibitory FcRs. This targeting induced CD4+ and CD8+ T cell responses independent the receptor's type. Moreover and in contrast to DEC205 and DCIR2 targeting, especially antigen delivery to FcγRIV was superior in inducing simultaneously CD4+ and CD8+ T cell responses, not only in a transgenic setting, but also in naïve mice. As FcγRIV is expressed on both splenic cDC subsets, we used it to verify the subset intrinsic preferences to trigger either $CD4^+$ or $CD8^+$ T cell responses. Thereby we could clearly show the induction of $CD4^+$ T cell responses by splenic $CD8^-$ DCs, whereas the $CD8^+$ DCs induced $CD8^+$ T cell responses.

The naïve $CD8^+$ T cell responses were of functional relevance, as we demonstrated the effective dose-dependent killing of peptide loaded target cells *in vivo*. Therefore, we suggest antigen targeting to FcRs as useful tool to induce *de novo* as well as the modulation of immune responses for future therapeutic applications. Additionally, we could demonstrate the responses to be effective in a murine melanoma model (in a preventive as well as a therapeutic setting). We now further investigate, which mechanisms play a role after antigen targeting to $CD11b^+CD8^-$ DCs, which adjuvant is most promising, and if the concomitant induction of a $CD4^+$ T cell response is beneficial to the anti-tumor $CD8^+$ T cell response in our system.

This project was partly funded by the DFG (RTG1660, RTG1962, CRC1181-TPA7, CRC1054-TPA6, and SO1149/1-1), BayGENE, and the Emerging Fields Initiative (BIG-THERA). This work was further supported by intramural funding (IZKF-J54, IZKF-A65, and IZKF-A68).

Abstract No. P31

Differential activation of human leukocytes by IgG immune complexes

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Immunoglobulin G (IgG) antibodies are known to interact with their receptors – the FcgR – mainly when in complex with their respective antigen. In previous studies we primarily focused on analyzing this interaction using cell lines expressing individual FcgRs. By generating IgG immune complexes from TNP-specific IgG and TNP-coupled BSA at different ratios we were for instance able to demonstrate a significant impact of immune complex size on FcgR interactions.

In the present study we extended our analysis of IgG immune complex binding towards human primary immune cells. We were thereby able to confirm the subclass- and immune complex size dependent binding of IgG to distinct FcgR expressing immune cell subsets. As expected, IgG1 and IgG3 immune complexes showed the strongest binding to all immune cells which could be further enhanced by increasing immune complex size. In contrast, large IgG2 and IgG4 immune complexes were exclusively able to interact with B cells and classical monocytes but not neutrophils, NK cells or non-classical monocytes.

We additionally measured calcium release and production of cytokines and further identified a differential activation of immune cells. First of all, increased immune complex size typically also enhanced cell activation. However, a size-independent pronounced cell activation could be observed especially for IgG1 immune complexes. Upon immune complex binding classical monocytes predominantly produced IL1b and TNFa. Interestingly, none of the studied cytokines could be detected in neutrophils despite of a strong calcium release response.

Since human primary monocytes and neutrophils express different combinations of FcgRs we also investigated the participation of individual FcgRs in immune complex binding and cell activation. By blocking FcgRs prior to immune complex incubation we were able to identify FcgRIIA as the sole FcgR responsible for IgG binding and subsequent activation of monocytes despite co-expression of FcgRIA (classical monocytes) or FcgRIIIA (non-classical monocytes). Furthermore neutrophilic granulocytes required FcgRIIIB for immune complex binding while cell activation was mediated by FcgRIIA.

Abstract No. P32

A VHH-assisted study of the C5 inhibitory mechanisms of OmCI and RaCI

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Complement component C5 has been targeted with various therapeutics, most notably by eculizumab, a monoclonal antibody that prevents cleavage of C5 into the anaphylatoxin C5a and C5b, the initiating building block of the membrane attack complex. Two species of tick, Ornithodoros moubata and Rhipicephalus appendiculatus, have evolved salivary proteins which inactivate C5 when secreted during feeding. The two proteins, dubbed OmCI and RaCI, after their respective species of origin, bind at sites distant from the eculizumab binding site on C5, potentially indicating they achieve efficacy through an allosteric mechanism. We sought to generate structural and biophysical data to understand the processes of conformational inactivation employed by immune evasion molecules, to inform the development of second generation therapeutics. Our approach utilised antibodies, which are established tools for structure-based drug discovery. Antibodies can be used to probe functional sites on proteins and may be employed as crystallographic chaperones; stabilising relevant conformations and mediating new crystal contacts to lower the energy barrier for transition into the crystalline state. Additionally, the diversity of antibody formats across species also presents opportunities for drug discovery and design. We produced immune libraries of anti-C5 VHH by performing Llama immunisations with C5. Phage display was used to create three sub-libraries, through panning on apo C5, C5-OmCI and C5-RaCI. We present non-competitive antagonists of the C5-RaCI complex; conformationally selective VHH which stabilise C5 conformations incompatible with the RaCI bound ensemble. Our VHH suggest that RaCI perturbs the conformational ensemble of C5, potentially more so than OmCI, and may achieve efficacy as a non-competitive antagonist of either C3b or the C5 convertase. Our VHH will be used as crystallographic chaperones to study the mechanisms of conformational activation and inactivation of C5.

Abstract No. P33

In utero exposure to anti-Aquaporin-4 antibodies alters brain vasculature und results in dysfunction of spatial cognitive function

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Neuromyelitis optica (NMO) is a neurological autoimmune disease, characterized by AQP4-IgG. The vast majority of patients harbor antibodies to Aquaporin-4 (AQP4), the water channel expressed on astrocytic endfeet surrounding blood vessels. AQP4-IgG leads to tissue damage primarily through complement mediated cytotoxicity (CDC). AQP4-IgG has been associated with fetal loss and may contribute to neurodevelopmental impairment in the offspring of patients with NMO. In order to study the effect of maternal AQP4-IgG on fetal development we *injected* intravenously a human monoclonal AQP4-IgG or an isotype matched control antibody to pregnant mice on embryonic day E14.5. A single exposure to AQP4-IgG during embryogenesis alters the brain vasculature of the in utero exposed male mice. The vasculature abnormalities persist throughout adulthood. We performed a PET study in these mice, which revealed BBB impairment most strikingly in the entorhinal cortex, which was accompanied by an increased blood flow. We showed that maternal AQP4-IgG binds to radial glia cells in the developing brain and impairs their role in vasculature remodeling. In order to investigate the effector mechanisms of AQP4-IgG on the fetal brain we injected AQP4-IgG into pregnant C1q Knockout mice. C1q knockout mice exposed in utero to AQP4-IgG did not show any vascular changes at embryonic day E18.5, suggesting that maternal AQP4 IgG acts through CDC. This led us to further study mutant antibodies that lack CDC. Four of the five mutant antibodies showed limited transplacental transport in vivo, which was associated with altered FcRn binding. One mutant exhibited normal transport across the placenta and can be used to further investigate effector mechanisms of AQP4-IgG on the fetal brain. Our study also suggests therapeutic approach to pathologic maternal antibodies using mutant Fc as a carrier to deliver decoy antigen to a pregnant woman, while sparing the developing fetus.

Abstract No. P34

Novel technologies for cloning of genes and expression of proteins

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Standard cloning methods and common protein expression systems show several limitations and constraints. Here we present novel cloning technologies for the fast and easy assembly of complex genetic constructs together with tools for temporally and spatially controlled protein expression in different expression systems. Furthermore, timely and spationally regulation of gene expression is possible as well as the regulation of cells inherent metabolism. Thereby, complex genetic and metabolic changes can be implemented into the cells of your interest.

Abstract No. P35

A Multivalent Binding Model Predicts FcγR Regulation and Effector Cell-Elicited Killing

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Immunoglobulin (Ig)Gis acrucial immune response regulator and particularly versatile therapeutic agent. These capabilities are due to itshigh-affinity antigen binding and its ability to direct immuneeffector cell-elicited responses via theFcγ receptors. IgGs elicit effector response through the multiple members of theFcγR family,cell types (e.g., macrophages, monocytes) and processes(e.g., ADCC, ADCP). Many possible design parameters—relative affinity towardFcγRs, responder cell populations, and antigen binding properties—make precisely predicting and manipulating effectorresponsean elusive goal.Here, we show that a model of multivalent receptor-ligand binding accurately accounts for the contribution of IgG-FcγR affinity and immune complex (antibody-antigen complex) valency [1].Modeling the binding of various effector cell types based on theirFcγR expression is better able to predict effector-elicited clearance of B16F10 melanoma cells or platelets than the receptor affinities directly. Moreover, the model accurately identifies and relies on binding to experimentallyverified critical effector populations.Building upon this model, we explore the predicted effects of IgG Fc combinations, and under which regimes two IgG isotopes or glycosylation forms might operate synergistically or antagonistically.In total, these results enable both rational immune complex design for a desired IgG effector function and the deconvolution of effectorcell-elicited responses.

[1]Ryan A. Robinett,Ning Guan,Anja Lux,Markus Biburger,Falk Nimmerjahn,Aaron S. Meyer.Dissecting FcγR Regulation through a Multivalent Binding Model. Cell Systems. 2018.

Abstract No. P36

Computationally designed repertoires of epitope-targeting nanobodies

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The potency of antibodies as therapeutics relies on their ability to recognize and block protein active sites with remarkable selectivity and affinity. However, even state-of-the-art methods for antibody discovery cannot be generally used to focus antibodies to a specific surface on the antigen, and retrospective epitope validation can be tedious and lead to a dead end. We develop a method for computational design of high-affinity site-specific camelid antibody (nanobody) repertoires. We recently demonstrated new methods for designing high-specificity and -affinity binders starting from a natural binding pair[2]. Here, we extend these methods for the design of libraries comprising millions of nanobodies with diverse CDR conformations and sequences, each of which is individually optimized to bind an arbitrarily chosen target site. This method should enable high affinity binder discovery in one shot to potentially any antigen of choice starting from its experimental structure, circumventing the need for tedious experimental affinity maturation and target-site verification.

[2] Netzer, R. *et al. Nat. Commun*. (2018)

Abstract No. P37

Structural insights into complement component C1 interaction with antibodies

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The human complement system provides an immediate and potent immune defense against microbial infection. It is also involved in tissue homeostasis affecting tumor growth, neurodevelopment, and diverse diseases have now been linked to complement activity. C1 (complement component 1) is the initiating complex of the so-called classical pathway of complement, consist of the recognition molecule C1q and the tetrameric protease complex C1r2s2. The activation of C1 is triggered when it recognises clustered antigen-bound immunoglobulins (IgM or IgG) on cell surfaces, acting as "danger signals". Binding to these multimeric antibody-antigen complexes induces conformational changes in C1 that lead to proteolytic activation of the two associated C1r and C1s proteases, thus allowing initiation of thecomplement cascade. *Despite its importance, we still do not know how this process occurs. This is in part to lack of high resolution structures of C1 complexes, which still remains challenging given its conformational heterogeneity. Here, we present the results of our current efforts to visualise in detail C1-antibody complexes, and resolve conformational steps in the activation process. The results are discussed in the context of previous data generated by our group.*

Abstract No. P38

DuoHexaBody-CD37, a novel bispecific antibody with a hexamerization-enhancing mutation targeting CD37, demonstrates superior complement-dependent cytotoxicity in pre-clinical B-cell malignancy models

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CD37 is a tetraspanin plasma membrane protein abundantly expressed on B cells that recently received renewed interest as a therapeutic target for the treatment of B-cell malignancies. Although complement-dependent cytotoxicity (CDC) has proven to be a powerful mechanism of antibody-mediated tumor cell killing in hematological cancer cells, CD37 antibody-based therapeutics currently in clinical development are poor inducers of CDC. We have developed DuoHexaBody-CD37, a novel humanized IgG1 bispecific antibody with an E430G hexamerization-enhancing mutation targeting two different CD37 epitopes that induces highly potent CDC activity in pre-clinical B-cell malignancy models. The E430G hexamerization-enhancing IgG Fc mutation was previously shown to improve the natural process of antibody hexamer formation through intermolecular Fc-Fc interactions between IgG molecules after cell surface antigen binding, thereby facilitating more efficient C1q binding and complement activation. The E430G mutation was introduced into humanized CD37 monoclonal antibodies that bind non-overlapping CD37 epitopes. Different antibody formats, including the single antibodies, antibody combinations and bispecific antibodies were tested for their capacity to induce CDC and antibody-dependent cellular cytotoxicity (ADCC). DuoHexaBody-CD37 showed potent CDC activity in vitro over a range of different B-cell lines and ex vivo in patient-derived chronic lymphocytic leukemia (CLL) cells, which was superior to other antibody formats evaluated. In a CDC assay using tumor cells obtained from a relapsed/refractory CLL patient who received prior treatment with rituximab, ibrutinib and idelalisib, DuoHexaBody-CD37 induced almost complete lysis (84% lysis at saturating concentration), thereby outperforming the single HexaBody molecules (15% and 23% lysis) and the combination (57%). In addition to its potent CDC activity, DuoHexaBody-CD37 was also capable of inducing potent ADCC in vitro. Importantly, DuoHexaBody-CD37 showed potent and specific depletion of the B-cell population in human whole blood and induced significant inhibition of tumor growth in vivo in cell line-derived NHL and CLL mouse xenograft models at doses as low as 0.1 and 1 mg/kg ($p<0.05$), respectively. These encouraging pre-clinical results suggest that DuoHexaBody-CD37 may serve as a potential therapeutic antibody for the treatment of human B-cell malignancies.

Abstract No. P39

Sorting of single-chain IgG secreting llama B cells using droplet-based microfluidics

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Llama produce classical IgG (heavy + light chain) and single-chain IgG (ScIgG; i.e. heavy chain only). The variable region of ScIgG (VHH; 16 kDa) can be easily expressed as a nanobody in bacteria, is generally of high affinity and is thermostable. Conventional screening of antigen-specific VHH from immunized llama involves phage display that is laborious and time consuming. Droplet microfluidics could be an alternate to phage display, being a fast, low cost and high-throughput method to encapsulate and assay single cells into compartments.

Aim: develop a pipeline to screen and sort antigen-specific ScIgG-secreting llama cell within 1 day of blood sampling from a llama.

Method: create a double fluorescent sandwich ELISA assay in 50pL droplets containing one cell or less. This assay is based on creating a physical surface inside the cell (line of magnetic beads (beadline) coupled to a ScIgG capture reagent), an anti-llama IgG labeled with red fluorescence, and the target antigen labeled with green fluorescence. If the cell in the droplet produces ScIgG, it will be absorbed by the beadline and the red fluorescence will relocalize onto this beadline. If this ScIgG is specific for the target antigen, the green fluorescence will also relocalize onto the beadline. Flowing these drops through a sorting chip scanned by a laser/PMTs, droplets containing relocalized red and green signals are sorted.

Results: llama were immunized with the sGP protein antigen from Ebola Virus as a proof of concept. Llama blood samples were taken 7 days after the third immunization. Cells were encapsulated and circulating ScIgG-secreting cells specific to sGP could be detected.

Conclusions: The pipeline works.

Perspective: After sorting, cells should now be recovered for DNA library preparation and sequencing. This pipeline should be advantageous for identifying nanobodies for diagnostic and (perhaps) even therapeutic applications.

Abstract No. P41

Complement alone drives efficacy of a chimeric anti-gonococcal monoclonal antibody

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Multidrug-resistant *Neisseria gonorrhoeae* is a global health problem. Monoclonal antibody (mAb) 2C7 recognizes a gonococcal lipooligosaccharide epitope that is expressed by >95% of clinical isolates and hastens gonococcal vaginal clearance in mice. Chimeric mAb 2C7 (human IgG1) with an E430G Fc modification that enhances Fc:Fc interactions and hexamerization following surface-target binding and increases complement activation (HexaBody® technology) showed significantly greater C1q engagement, C4 and C3 deposition compared to mAb 2C7 with wild-type Fc. Greater complement activation translated to increased bactericidal activity *in vitro* and consequently, enhanced efficacy in mice, compared with 'Fc-unmodified' chimeric 2C7. Gonococci bind the complement inhibitors factor H (FH) and C4b-binding protein (C4BP) in a human-specific manner, which dampens antibody-mediated complement-dependent killing. 2C7-E430G Fc overcame the barrier posed by these inhibitors in human FH/C4BP transgenic mice, where a single 1 µg intravenous dose cleared established infection. Chlamydia frequently co-exists with and exacerbates gonorrhea; 2C7-E430G Fc also proved effective against gonorrhea in gonorrhea/chlamydia co-infected mice. Complement activation was necessary and sufficient for 2C7 function, evidenced by: i) 'complement-inactive' Fc modifications that engaged FcγR rendered 2C7 ineffective; ii) 2C7 was non-functional in $C1q^{-/-}$ mice or when C5 f unction was blocked and iii) 2C7 remained effective in neutrophil-depleted mice and in mice treated with PMX205, a C5a receptor (C5aR1) inhibitor. We highlight the importance of complement activation for anti-gonococcal antibody function in the genital tract.

Abstract No. P40

B cell-autonomous antibody engineering

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B cells exhibit a great potential for genetic engineering due to their ability to secrete antibodies that help to protect the host from infections. With the advent of CRISPR/Cas9, it is now possible to edit the genome of primary human B cells, thereby exploiting these engineered cells as production factories for therapeutic proteins as well as endowing them with reprogrammed antibody specificities. Many studies, however, have reported immunogenic side effects as well as Cas9 off-targets leading to mutations in the genome that are potential drivers of cancer. Here we aim to investigate and exploit natural DNA-breaks induced by the B cell intrinsic- enzyme activation-induced cytidine deaminase (AID) as target sites for engineering of the antibody heavy chain gene. Our preliminary data suggest that nucleofected DNA substrates can be successfully integrated into the antibody switch region of activated primary human B cells without the need for extrinsic nucleases. Integration of a substrate comprising an entire exon with flanking intronic sites led to its successful splicing into antibody mRNAs. Hence, we now aim to maximize engineering efficiencies by optimizing B cell stimulation, DNA substrate design and splicing of integrated exons to foster efficient expression of the engineered antibody. Besides developing an alternative to current engineering techniques, we are aiming to use our system to produce high-affinity antibodies harbouring pathogen-specific binding elements bearing potential for future vaccination strategies and therapeutics.

Abstract No. P42

Characterization of the human antibody repertoire following immunization with serogroup B meningoccoccus 4CMenB vaccine

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Neisseria meningitidis serogroup B (MenB) is a major cause of sepsis and invasive meningococcal disease, against which a multicomponent vaccine with broad coverage, 4CMenB, has been approved. The shaping of the B cell repertoire induced by vaccination is a fundamental aspect that leads to protective immunity and needs to be explored in response to a multicomponent bacterial vaccine. To gain a comprehensive picture of the antibody response induced upon 4CMenB vaccination in humans, we performed a translational study exploring the antibody response to each of three main recombinant protein antigens included in the 4CMenB vaccine. A panel of antigen-specific monoclonal antibodies obtained from adult vaccinees has been isolated and extensively characterized both as Fab fragments and as full length IgG1 mAbs. We identified monoclonal antibodies targeting multiple epitopes on each antigen and found that some of them were cross-reactive to antigen variants different from the ones present in the vaccine, thus conferring capability to target a broader range of bacterial strains. Interestingly, synergy between antibodies recognizing different epitopes enhanced the potency of the bactericidal response. A longitudinal study on the pattern of recombination and mutations occurring in BCRs at different time points after vaccination is currently ongoing to monitor the key features of clonally expanded antigen-specific B-cells. The identification of a peculiar Ig gene usage linked to the development of a functional long-lasting response against MenB antigens in different vaccinees could lead to the definition of a BCR signature specifically induced by 4CMenB vaccination. This study shed light on mechanistic nature of protective immunity induced by 4CMenB vaccine.

Abstract No. P43

Extrinsic Sialylation of Immunoglobulin G and its effect on therapeutic antibodies

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Glycosylation of Immunoglobulin (IgG) plays an important role for IgG effector functions. Thus, afucosylated IgG glycovariants have an enhanced cytotoxic activity, whereas IgG glycovariants rich in terminal sialic acid residues show an anti-inflammatory effect. More recent evidence suggests that terminal α2,6 linked sialic acids can be attached following IgG secretion by plasma cells. These findings raise concerns for the use of therapeutic antibodies as they may change their glycosylation status in the patient. To investigate if B cell extrinsic sialylation modifies therapeutic IgG preparations, we injected $\mu MT^{-/-}$ mice lacking mature B cells, as well as $ST6Gal1^{-/-}$ mice, lacking the Sialyltransferase 1 which catalyzes the addition of α2,6 linked sialic acid residues, with IVIg or desialylated IVIg. IgG Glycan analysis of mouse serum of several consecutive days after IVIg administration was performed by UPLC analysis. Our study suggests that IVIG glycosylation is stable upon injection *in vivo*. If at all, only a very small fraction of IgG molecules acquired sialic acid structures, which will not impact therapeutic IgG function.

Abstract No. P44

Generation of monoclonal antibodies specific for human pancreatic secretory zymogen-granule membrane glycoprotein 2

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Autoimmune diseases are failures of the immune system leading to attacks against own body structures. One example is Crohn's disease (CD). Studies identified the pancreatic secretory zymogen-granule membrane glycoprotein 2 (GP2) as the auto-antigenic target for CD. In order to establish suitable diagnostic tools for monitoring the diseases, monoclonal antibodies (mAbs) are needed. Human GP2 is produced as a long and a short isoform by alternative splicing. As the abundance and ratio of long and short isoform seem to be related to CD and its progression our goal is the generation of antibodies, which are able to distinguish between the two isoforms.

Standard immunization with recombinant GP2 (long or short isoform) followed by hybridoma technology or phage display resulted in antibodies binding either to both isoforms or were specific for the long isoform (mAbs only). These results are certainly due to the high amino acid sequence identity. As there are no crystal structures available for GP2 we performed bioinformatic prediction of short GP2 with regard to possible antigenic epitopes. We identified a unique sequence (SCAR) with possible surface accessibility at the splicing position. For the induction of specific mAb responses, we used two versions of immunoconjugates. For VP-SCAR1 the peptide sequence was conjugated chemically to a viral coat protein. For VP-SCAR2 the SCAR-DNA was cloned together with the DNA of the viral coat protein and expressed as recombinant chimeric version. The immunization with VP-SCAR1 led to GP2 specific serum titers (OD450nm>0.4). We used our novel selection technology to select hybridoma cells producing short GP2 specific antibodies. Within this technology, B-lymphocytes are fused with transgenic myeloma cells, which can be captured via the presence of the produced antibody on the cell surface. Hybridoma cells secreting short GP2 specific antibodies were selected using fluorescently labeled short GP2. We successfully selected 3.768 (high signal) and 132.129 (medium signal) hybridoma cells. Different immunoassays were carried out. GP2 short specific signals (OD450nm~0,45) were detected only in polyclonal cultures. So far, these positively tested hybridoma cells seem to lose the ability to produce a stable short GP2 specific antibody, after passaging.For further investigations, we will use VP-SCAR2 for immunization and compare the outcome of positive clones to VP-SCAR1. The generation of specific Abs for the short GP2 isoform so far was not possible by standard hybridoma technology or phage display. Using our alternative immunization method and novel selection strategy we hope to overcome these limitations.

Abstract No. P45

Pneumococcal vaccination elicits public B-cell repertoires and serum antibody clonotypes derived from the IgM+IgD+CD27+ memory B cell compartment

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Increased susceptibility to infections with encapsulated bacteria (e.g., *Streptococcus pneumoniae*) whose cell walls are surrounded by a protective layer of polysaccharide (PPS) is a hallmark of primary antibody deficiency as well as immunosenescence. Protective anti-PPS antibodies promote phagocytosis and thus reduce/block infection. While considered to be T independent, antibodies elicited by Pneumovax®23 (PPSV23) are class switched; and the genesis of these IgG Abs remains unclear even after decades of study. In order to develop a framework for the comprehensive analysis of the human vaccine response to PPSV23, we have used a combination of multi-parameter flow cytometry, deep sequencing of the VH repertoire of Day 7 memory B cell subsets and native VH:VL cDNA pairs of responding plasmablasts (VH:VL BCR-seq), and mass spectrometry to determine dominant IgG clonotypes comprising the endpoint serological protein antibody repertoire (Ig-seq). In a pilot study of the human vaccine response to the 6B polysaccharide component of PPSV23 in a healthy 55 y/o woman, we determined that of the 10 most prevalent IgG clonotypes, representing >80% of the observed CDR-H3 peptides, two mapped to the $IgM^{+}IgD^{+}CD27^{+}$ compartment and one demonstrated convergence to a previously published anti-PPS antibody. Convergent sequences were also found in three additional study subjects. There remain large gaps in our understanding of the underlying mechanisms by which the commonly used vaccine Pneumovax®23 generates protective antibodies. Our findings should help set the stage for a better understanding of how to elicit protective antibody responses to encapsulated bacteria in the elderly and in patients who suffer with humoral immune deficiencies.

Abstract No. P46

Inhibition of Ab-complex mediated inflammation by recombinant IgG1 Fc hexamer

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High-dose immunoglobulin (IVIG / SCIG) is used to treat autoimmune diseases. The Fc portion of the IgG molecule has been suggested to be critical for many of the clinically beneficial effects seen in IVIG. This includes scavening of complement and blockade of Fcγ receptors (FcγR). Recent data indicate that recombinant Fc (rFc) multimers exhibit potent anti-inflammatory properties. In this study, we investigated the inhibitory properties of a rFc hexamer (termed Fc-µTP-L309C) generated by fusion of the IgM µ-tailpiece to the C-terminus of human IgG1 Fc on FcγR effector functions and the complement system. In-vitro, the Fc-µTP-L309C demonstrated high avidity binding to C1q and FcγR receptors. Consequently, Fc-µTP-L309C potently inhibited full activation of the classical pathway (CP) and FcγR effector functions such as e.g. ADCC. In a rat model of acute neuromyelitis optica (NMO), administration of Fc-µTP-L309C reduced brain tissue damage and deposition of C5b-9. Similarly, in a mouse model of collagen Ab-induced arthritis Fc-µTP-L309C reduced clinical manifestations along with reduction of C3 and C5a levels in joint washes. Inhibition of the classical pathway in-vivo seemed to be predominantly mediated by consumption of C1q. We conclude that interference with activation of CP may be an important protective mechanism of Fc-µTP-L309C in Ab-complex mediated diseases

Abstract No. P47

A new model of inducible neutropenia confirms neutrophil contribution to two different antibody-mediated inflammatory reactions in mice

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Neutrophils have extensively been described as key drivers of inflammatory reactions, based on their rapid arrival in high numbers at sites of inflammation, their release of potent inflammatory mediators and their capacity to attract numerous other immune cells.

Until now, most approaches to study neutrophil contribution to disease were based on the use of neutrophil-depleting antibodies. While this approach accelerated neutrophil research, one has to keep in mind possible off-target effects of these antibodies. Especially in the context of antibody-dependent reactions the interpretation of results may be challenging when depleting antibodies have been used.

To assess the role of neutrophils during antibody-induced inflammatory reactions, we developed a mouse model (PMN_{DTR}mice), in which neutrophil-specific expression of the diphtheria toxin (DT) receptor is controlled by the hMRP8 promoter. Following systemic injection of DT, neutrophils were selectively and efficiently depleted (Reber, Gillis *et al.* J Exp Med 2017). We then evaluated the contribution of neutrophils in two models of antibody-induced inflammatory reactions using PMN_{DTR}mice and depleting antibody-based approaches.

Here, we compared two antibody-induced inflammatory reactions: 1.**Acute airway inflammation**was triggered by concomitant intravenous injection of ovalbumin (OVA) and intranasal administration of OVA-specific antiserum. After 18 hours, blood leakage into the broncho-alveolar fluid and leukocyte infiltration in the airways were evaluated. Neutrophil depletion by either of the tested approaches abolished these symptoms; 2.**K/BxN arthritis**was induced by the transfer of anti-glucose-6-phosphate isomerase (GPI) antibodies-containing serum from K/BxN mice into recipient mice. Transiently developing arthritis, characterized by a swelling of the ankles and the infiltration of inflammatory cells into the joints was significantly reduced in neutropenic mice independently of the depletion strategy used.

Thus, PMN_{DTR}mice are a valuable new tool for the study of neutrophil contribution in mice. We could validate its appropriateness in both disease models tested,in which weconfirmed the pro-inflammatory role of neutrophils. Additionally, this model has valuable advantages: it is not expected that the use of DT affects Fc receptor biology, while most depleting antibodies depend on Fc receptor engagement. As a consequence, it can be difficult to evaluate, which proportion of the observed effects of neutrophil-depleting antibody treatment is due to the actual lack of the neutrophils and which proportion to Fc receptor occupancy or downregulation. Furthermore, this model allows us to perform adoptive transfers of DT-insensible neutrophils inPMNDTRmice, opening new possibilities for the study of neutrophils in vivo.

Abstract No. P48

Human IgA coat a wide range of gut commensals

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In humans, several grams of IgA antibodies are secreted every day in the intestinal lumen harboring an extremely dense consortia of symbionts. Mice secrete only one IgA isotype that was recently described as polyreactive against a broad pattern of commensals, but the type of relations human IgA establish with the microbiota remain elusive. Furthermore, humans secrete two different IgA isotypes, and it remains unknown whether different symbionts would be preferentially bound by IgA1 or IgA2. Here, we determined the reactivity profile of native human IgA monoclonal antibodies to commensals and also compared binding patterns of both polyclonal IgA sub-classes. We found that monoclonal IgA were polyreactive and bind a diverse, but nevertheless restricted, subset of gut commensals. These antibodies harbored high numbers of somatic mutations suggesting an antigen-mediated selection. Most commensals are dually targeted by IgA1 and IgA2 in the small intestine, but a distinct subset of colonic bacteria was preferentially coated by IgA2 alone. IgA1 and IgA2 anti-carbohydrate repertoires also exhibited distinct binding patterns. Altogether, our data indicate that, although bearing signs of affinity maturation, human IgA are clearly microbiota-polyreactive at clonal level. Importantly, each monoclonal IgA studied (n=16), exhibited a private microbiota binding pattern. Finally, IgA2 has broader reactivities, compared to IgA1, although both isotypic repertoires are largely overlapping.

Abstract No. P49

The molecular pathways of IgG oligomerization and classical complement activation on antigenic surfaces

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The human immune system consists of powerful defense mechanisms that recognize and eliminate pathogens from our bodies. Immunoglobulin G (IgG) antibodies play a central role in this protection by alerting and activating components of the innate immune system, such as the complement cascade. Here, we employed high-speed atomic force microscopy [1, 2] to conclusively show that IgGs oligomerize on antigenic surfaces via two interconnected Fc-mediated pathways. Bivalently binding IgG molecules were recruited from solution via the formation of Fc-Fc interactions with membrane-bound antibodies, whereas monovalently binding variants [3] also assembled into oligomers through lateral diffusion. All intermediates of the oligomerization process were observed, although only full hexamers bound C1q. Complement dependent cytotoxicity (CDC)-enhancing mutations [3] increased the abundance of higher-order oligomers, while mutations that were shown to decrease CDC also hindered the hexamerization process. Functional assays on cells confirmed that IgG hexamerization was required for maximal complement activation, and that dimers, trimers and tetramers were mostly inactive. We present a dynamic IgG oligomerization model, which provides a framework for immunotherapy optimization and for exploiting the macromolecular assembly of IgGs on antigenic surfaces [4].

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Abstract No. P50

Development of C5a-binding biologics for the immunomodulation of anaphylatoxin signalling pathways

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The signalling pathways mediated by the anaphylatoxins complement component 3a and 5a (C3a and C5a) have been classically regarded as contributing to proinflammatory responses. While in the context of infection biology C5a-mediated neutrophil chemotaxis is seen as an integral part of the immune system defence against pathogens, in cases of immune complex disease, ischaemia/reperfusion injury or allergic asthma and bronchoconstriction, the outcome of C5a stimulation for patients may be less benign. Hence, the development of antibodies and antibody-like molecules for the pharmaceutical immunomodulation of anaphylatoxin-mediated signalling pathways will require substantial optimisation to strike the proper balance between potency (e.g. C5aR1 signalling inhibition), disease entity (e.g. allergic asthma), timing and dosing, as well as the precise nature of the targeted molecule (e.g. anaphylatoxin receptors vs the anaphylatoxins themselves). The development of high-affinity, precisely targeted and safe antibody-based therapeutics is, therefore, a prerequisite for the efficient management of human diseases associated with anaphylatoxin pathways. To this end, we are developing antibodies that recognise recombinant and authentic human and mouse C5a, inhibit C5a-induced chemotactic responses in neutrophils and macrophages and show nM affinity and adequate kinetic dissociation parameters. We have tested the properties of several anti-C5a antibodies using human granulocytes purified from EDTA venous blood samples. We are also seeking to understand better the immunoevasive virulence factors that interfere with anaphylatoxin signalling pathways to enhance the usefulness and efficacy of these novel antibodies. Disrupting C5a signalling in the right context and at the right time may bring favourable clinical outcomes for patients. The development of the next-generation C5a and C3a immunomodulatory antibodies must take into consideration the large and growing literature on the pleiotropic effects of anaphylatoxins if they are to become useful and safe medicines.

Abstract No. P51

Development of Antibody Therapeutics against Flaviviruses using Plant Produced ZIKV-NS2b antigen

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Zika virus (ZIKV) has become an emerging virus since its first major outbreak in the South Pacific, 2007. It is one of the most important human viral pathogens among the Flaviviridae family, which also include: Hepatitis C virus (HCV), Dengue virus (DENV), West Nile virus (WNV) and Yellow fever virus (YFV). The recent emergence of ZIKV has posed serious threats to human health worldwide due to the high number of people infected mainly in South America, Central America and or Caribbean. The disease presents different characteristics in relation to the other flaviviruses including, fetal anomalies (microcephaly), neuromotor disorders (Guillain-Barré syndrome); has also been associated with transplacental infection. Zika's disease is mainly spread to humans by the bite of infected *Aedes aegypti* or *Aedes albopictus* mosquitoes. According to data from the Brazilian Ministry of Health, in 2016 there were more than 130,000 confirmed cases of the disease in the country and more than 200 children with microcephaly. In July 2016 the WHO published a Strategic Response Framework to Zika as an emerging disease. It is necessary to produce vaccines against ZIKV, as well as new diagnostic systems more accurate and accessible to the population. The present work demonstrated the possibility of producing recombinant NS2b protein of ZIKV using plant as bioreactor with the purpose of obtaining vaccine candidate proteins. Protein was expressed transiently in leaves of *Nicotiana benthamiana* L. via Agrobacterium tumefaciens. To facilitate the purification of the recombinant protein a tail fusion of Hydrofobin was used. The protein was purified and used in the immunization of rabbits and mice for the production of monospecific antibodies. Serum antibodies from patients who were infected with ZIKV and antibodies produced in the animals specifically recognized the protein, which allowed us to conclude that the NS2b protein has potential for diagnosis as well as for vaccine formulation.

Abstract No. P52

The novel therapeutic DuoHexaBody-CD37 induces efficient killing of tumor B-cells ex vivo via complement-dependent cytotoxicity, even in relapsed and/or refractory patient samples

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CD37 is a tetraspanin molecule expressed on mature B-cells, but absent on normal stem cells and plasma cells. Due to this cellular distribution, which is highly similar to CD20, CD37 has gained attention as a target for B-cell lymphoma, especially for rituximab-resistant and relapsed patients.

The CD37-targeting antibody-based products that are currently being evaluated in the clinic are generally poor inducers of complement-dependent cytotoxicity (CDC) – a rapid and powerful Fc-mediated effector function. DuoHexaBody-CD37 is a bispecific IgG1 antibody targeting two non-overlapping epitopes in the large extracellular loop of CD37, with a hexamerization-enhancing mutation (E430G) in the IgG Fc domain. The E430G mutation increases the intrinsic capacity of IgG1 molecules to form hexameric antibody clusters upon binding to membrane-bound antigens, thereby facilitating binding of the hexavalent complement component C1q and subsequent CDC activity (Diebolder et al., Science 2014; de Jong et al., PLoS Biology 2016). The combination of dual epitope targeting and enhanced hexamerization of DuoHexaBody-CD37 upon binding to the cell surface significantly increases complement activation and CDC efficacy in CD37-positive B-cells.

To assess its clinical relevance and potential, we tested the capacity of DuoHexaBody-CD37 to induce *ex vivo* CDC activity using a broad range of B-cell lymphoma and leukemia patient samples (Diffuse Large B-Cell Lymphoma, Follicular Lymphoma, Mantle Cell Lymphoma, Marginal Zone Lymphoma, Chronic Lymphocytic Leukemia, n=40) derived from both newly diagnosed (ND) (n=23) and relapsed and/or refractory (RR) patients (n=17), most of whom had received prior treatment with rituximab. Our results revealed that, independent of the B-cell malignancy subtype, DuoHexaBody-CD37 induced potent CDC activity with a median lysis of 82% (n=40, both RR and ND) at 10 μg/ml. Furthermore, CDC induced by DuoHexaBody-CD37 was shown to be less sensitive to the expression of complement regulatory proteins (CD46, CD55, CD59), compared to the tested CD20 antibodies, although the few patient samples that were resistant to DuoHexaBody-CD37 in the *ex vivo* CDC assays expressed significantly higher CD59 levels than the responsive samples.

Importantly, DuoHexaBody-CD37 was equally effective in inducing CDC in samples derived from ND and RR patients that received prior treatment with rituximab.

In conclusion, our results indicate great therapeutic potential for DuoHexaBody-CD37 in B cell malignancies, based on its strong capacity to induce CDC, even in ex vivo samples from patients that had relapsed from or were refractory to rituximab-containing treatments.

Abstract No. P53

A mechanism to diversify antibodies by large DNA-insertions at AID-induced break sites

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Diversification of antibodies results from a complex process of somatic gene rearrangements and mutations. We recently found that the switch region of the immunoglobulin heavy chain (IgH) locus frequently carries large DNA inserts that stem from different chromosomes. As a functionally relevant example, the genomic integration of a *LAIR1* exon was detected in immunoglobulin genes of malaria-exposed individuals providing antibodies with a parasite-reactive extra domain. Studying the *IgH switch region as well as* antibody transcripts of various donors suggests that the incorporation of DNA-inserts is common in the general population and - although rare in each individual - can contribute to functional antibodies. Our data provide evidence that large DNA insertions mechanistically originate from DNA repair of AID-induced break sites. Therefore, we are studying insert origin and source, the break induction as well as the involvement of different DNA repair pathways. We developed an *in vitro* system to study AID-mediated breaks repair in primary human B cells enabling manipulation by repair inhibitors, knockdown and overexpression of genes. In addition, we study switch-insert frequencies in activated murine CH12 B cell lines with CRISPR/Cas9 knockouts as well as human patients with defects in DNA repair factors. In all cases, the switch region of the IgH locus is amplified by PCR in an unbiased way, analyzed by MinION sequencing and screened by a special bioinformatics pipeline. Our preliminary data show that insert incorporation is not species specific because it is also inducible in murine B cells. Moreover, inhibition of the classical non-end joining pathway may reduce the frequency of switch-insert acquisition. Studying the mechanism of insert incorporation in the antibody switch region will not only unravela new way of antibodies diversification but might also open new approaches for the engineering of B cells.

Abstract No. P54

The CIP Complement Inhibitory Protein from Streptococcus agalactiae at the interface between innate and acquired immunity

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Group B Streptococcus (GBS) colonizes the human lower intestinal and genital tracts and constitutes a major threat to neonates from pregnant carrier mothers and to adults with underlying morbidity. Complement effectors serve as first line of defense against GBS infection, promoting phagocytic killing of these bacteria by neutrophils and macrophages. The pathogen has evolved several mechanisms to inhibit Complement activation to colonize and invade its host. Among them, the polysaccharidic capsule containing sialic acid that covers the bacterial surface and interferes with C3b deposition and opsonophagocytic killing. We have identified a GBS secreted protein named Complement Interfering Protein (CIP) that downregulates Complement activation via the classical and lectin pathways but not the alternative pathway. CIP shows high affinity toward C4b and prevents the formation of the C4bC2a convertase. Addition of recombinant CIP to GBS cip-negative bacteria resulted in decreased deposition of C3b on their surface and diminished phagocytic killing in a whole-blood assay. We recently discovered that CIP can also interact with C3 and its C3b and C3d fragments. Immunoassay-based competition experiments showed that binding of CIP to C3d interferes with the interaction between C3d and the CR2/CD21 receptor on B cells. By B-cell intracellular signaling assays, CIP was confirmed to down-regulate CR2/CD21-dependent B-cell activation. The CIP domain involved in C3d binding was mapped via hydrogen deuterium exchange–mass spectrometry. CIP is therefore a new member of the growing list of virulence factors secreted by gram-positive pathogens that incorporate multiple immunomodulatory functions.

Abstract No. P55

A Novel Mechanism for Generating the Interferon Signature in Lupus: Opsonization of Dead Cells by Complement and IgM.

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OBJECTIVE:

In vitro studies suggest that the type I interferon (IFN) signature seen in most lupus patients results from Fcγ receptor-mediated uptake of nucleic acid-containing immune complexes by plasmacytoid dendritic cells and engagement of endosomal Toll-like receptors. The aim of this study was to reexamine the pathogenesis of the IFN signature in vivo.

METHODS:

Lupus was induced in mice by injecting pristane. Some mice were treated with normal immunoglobulin or with cobra venom factor to deplete complement. The IFN signature was evaluated by polymerase chain reaction. The IFN signature also was determined in C4-deficient patients and control subjects.

RESULTS:

Wild-type C57BL/6 mice with pristane-induced lupus developed a strong IFN signature, which was absent in immunoglobulin-deficient (μMT), $C3^{-/-}$, and $CD18^{-/-}$mice. Intravenous infusion of normal IgM, but not IgG, restored the IFN signature in μMT mice, and the IFN signature in wild-type mice was inhibited by depleting complement, suggesting that opsonization by IgM and complement is involved in IFN production. Consistent with that possibility, the levels of "natural" IgM antibodies reactive with dead cells were increased in pristane-treated wild-type mice compared with untreated controls, and in vivo phagocytosis of dead cells was impaired in C3-deficient mice. To examine the clinical relevance of these findings, we identified 10 C4-deficient patients with lupus-like disease and compared them with 152 C4-intact patients and 21 healthy controls. In comparison with C4-intact patients, C4-deficient patients had a different clinical/serologic phenotype and lacked the IFN signature.

CONCLUSION:

These studies define previously unrecognized roles of natural IgM, complement, and complement receptors in generating the IFN signature in lupus.

Abstract No. P56

Proteases C1r and C1s affect binding of C1q to certain human IgG subclasses

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Complement is an important effector mechanism for antibody-mediated clearance of microbes and altered host cells. When antibodies bind a target cell surface, the first complement protein C1 activates a proteolytic cascade that results in labeling of cells for phagocytosis, attraction of leukocytes and formation of lytic pores. The large C1 complex consists of the recognition protein C1q and its associated tetramer of serine proteases C1r and C1s ($C1r_2C1s_2$). C1q consists of a 'bunch' of six polypeptide chains that together bind IgM or Fc domains of clustered IgG's. Following binding of C1q to Ig, the associated $C1r_2C1s_2$ become activated to cleave C4.

While interactions between C1 and Ig molecules are believed to depend solely on the globular heads of C1q, we here found that C1r and C1s influence the binding of C1q to certain IgG subclasses. Even though $C1r_2C1s_2$ do not interact directly with IgG-Fc, we observe that they enhance binding of C1q to human monoclonal IgG1 and IgG2, but not IgG3 or IgG4 (observed on DNP-coated beads and bacterial surfaces). Using SPR,we find that C1r and C1s not only influence the association of C1q to surface-bound antibodies, but also stabilize the formed C1q-IgG complexesInterestingly, C1q binding to IgG1 and IgG2 in absence of $C1r_2C1s_2$ strongly improved when we introduced IgG mutations designed to enhance IgG-Fc clustering on the target surface, while C1q binding in presence of the proteases was hardly affected.

Finally we studied dissociation of C1q after binding IgG. Removal of $C1r_2C1s_2$ from surface-bound C1 by C1-inhibitor resulted in dissociation of C1q from IgG1 and IgG2 wildtype antibodies, but not from IgG1/2 Fc-mutants or IgG3 antibodies. This suggests that C1q bound to IgG3 and hexabody platforms could be 'recycled' by insertion of new proteases.

Altogether our data suggest that the $C1r_2C1s_2$ proteases affect the conformation of C1q and its subsequent interaction with antibody platforms. Finally these basic insights in antibody-complement interactions will improve our understanding of (un)wanted complement activation processes.

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