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Sialylated Autoantigen-specific Monoclonal Antibodies to Treat Autoimmune Diseases

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Abstract

In healthy higher organisms the immune system is in a tight balance between sensitivity to pathogens and tolerance of self-structures. In autoimmune diseases like rheumatoid arthritis this steady state is imbalanced. Immune cells and antibodies, which are meant for pathogen clearence are suddenly prone to attack self-structures and promote inflammation of healthy tissue. A standard model to study immunological mechanisms of rheumatoid arthritis is the chicken collagen-induced arthritis (CIA) mouse model, where the immune response is characterized by high titers of IgG antibodies reactive against self-collagen type II (CII), a protein component of the synovial fluid in joints. Autoreactive IgG's are potent mediators of inflammatory responses in self-tissues, but their effector function depends on the structure of one N-linked oligosaccharide in the effector region of IgG antibodies (Fc region). Recent findings have suggested that terminal sialic acid on this Fc-glycan provides antiinflammatory effector properties to IgG's. Desialylation leads to a shift and gives the same IgG antibodies pathogenic effector functions.

The goal of this diploma thesis was to investigate the therapeutic potential of antigen-specific sialylated IgG antibodies in CIA. We established a CIA mouse model, where we were the first to show an antigen-specific prevention from the development of arthritis with sialylated anti-CII IgG1 antibodies that were cloned from freely accessible sequences described in the literature. We could show that enzymatic glycosylation of antibodies switched the effector function from pro- to antiinflammatory. These findings strongly support the theory of antiinflammatory activities of sialylated recombinant IgG's, which could provide a potent future tool for

preventing the development of or even treat autoimmune diseases antigen-specifically.

Zusammenfassung

Im gesunden Organismus höher entwickelter Lebewesen bewegt sich das Immunsystem zwischen Sensibilität gegenüber Krankheitserregern und Toleranz gegenüber körpereigenen Strukturen. Bei Autoimmunerkrankungen wie Rheumatoider Arthritis gerät diese Balance aus dem Gleichgewicht. Immunzellen und Antikörper, die eigentlich für die Bekämpfung von Pathogenen zuständig sind, beginnen dabei körpereigenes Gewebe zu attackieren, wobei Entzündungsreaktionen hervorgerufen werden. Ein Standardmodell zur Untersuchung immunologischer Mechanismen bei Rheumatoider Arthritis ist das Hühnerkollagen-induzierte Arthritis (CIA) Mausmodell, dessen Krankheitsbild von hohen IgG-Antikörpertitern gekennzeichnet ist. Autoreaktive Antikörper richten sich dabei gegen körpereigenes Kollagen Typ II (CII) in der Gelenksflüssigkeit und rufen dort Gelenksentzündungen hervor. Dieser pathogene Mechanismus ist vor allem von der Glykosylierungsstruktur im Effektorteil (Fc) der IgG-Antikörper abhängig. Aktuelle Studien zeigen, dass terminale Sialinsäure an dieser Zuckerkette den Antikörpern entzündungshemmende Eigenschaften verleiht. De-sialylierte IgG Antikörper werden stattdessen mit inflammatorischen Effektorwirkungen in Verbindung gebracht.

Die Untersuchung des therapeutischen Potentials dieser sialylierten Antiköper bei CIA war Ziel meiner Diplomarbeit. Dazu etablierten wir ein CIA Mausmodell, an welchem wir erstmalig zeigen konnten, dass sialylierte Anti-CII-Antikörper die Ausdehnung von Arthritis verhindern konnten. Wir konnten zeigen, dass rekombinante Antiköper, deren Sequenzen in der Literatur frei zugänglich waren, durch enzymatische Glykosylierung ihre Effektorwirkung von entzündungsfördernd auf –hemmend wechselten. Diese Erkenntnisse untermauern die These, dass

sialylierte IgG-Antikörper entzündungshemmende Aktivitäten besitzen und somit zukünftig eine potente Waffe zur Prävention oder gar Behandlung von Autoimmunerkrankungen darstellen können.

1. Introduction

1.1. The Immune System

1.1.1. A Defense Machinery

The immune system of mammals provides a defence machinery against many kind of malignant influences that may harm the integrity of the organism. Besides toxins, foreign pathogens like viruses, bacteria and microbes are main targets of defence as well as abnormal self-structures like tumor cells of the host itself. The specificity of the immune system can potentially be directed against any molecular structure. But to avoid the problem that the immune system reacts against host self-structures, autoreactive components are deleted during developmental checkpoints. In healthy human individuals or animal models like mice the immune system is in a tight balance between sensitivity against pathogens and tolerance of self-structures, food and harmless non-self structures like environmental factors. [1]

1.1.2. Innate and Adaptive Immunity

The process of a normal immune response against pathogens is a 2-stage immune response characterized by the initial defence of components of the innate immunity, which is unspecific and triggered by general inherent recognition mechanisms of pathogen patterns, followed by an adaptive immune response, where immune cells are generated with high specificity against the one disease-causing pathogen. After pathogen clearance, some of these cells that specifically recognize this disease-

causing pathogen turn into memory cells to provide long-term immunity against one specific microbe.

1.1.3. Humoral and Cell-mediated Immunity

The two major branches of the adaptive immune system are the humoral immune response and the cell-mediated immune response. Depending on the kind of invading pathogen, which can be intra- or extracellular, there is preferentially one way of immune response triggered. While the cell-mediated immunity is characterized by cell-cell interactions, where immune cells like T-cells help killing infected or malignant host cells, the humoral immunity is provided by B-cells that upon stimulation with antigens and other immune cells differentiate into plasma cells, which secrete antibodies with defined specificities. Toxins, pathogens and molecular structures that are tagged by antibodies are prone to be erased by the complement system or by immune cells like macrophages, which can engulf and digest for example tagged bacteria. The most abundant antibody isotype in the blood is IgG. About 60% of IgG antibodies are circulating in the body via lymphatic and blood vessels, 40% are distributed in extracellular fluid. [1, 2]

1.1.4. Reaching Potentially Unlimited Specificity

B-cells can reach a vast number of specificities against molecular structures with B-cell receptors (BCR) and antibodies, which are secreted forms of BCR, though they have only a limited number of genes. The variable domains, which compose the antigen-binding site, are built up of 3 gene segments, the so-called VDJ-genes. During the VDJ-recombination event of heavy chains 1 of 40 V (variable) segments, 1 of 25 D (diversity) and 1 of 6 J (joining) segments are recombined together with the corresponding constant domains (**Figure 1**). Light chains are produced analogue but there are no light chain D-segments. This somatic recombination of DNA is carried out by RAG (recombination activating gene) enzymes leading to unique VDJ-recombinations in every B-cell. Another mechanism in fine-tuning the antigen-binding specificities is somatic hypermutation, a process where mature B-cells undergo mutations in the hypervariable regions of the V(D)J-gene segments. These sites

correspond with the complementary determining regions (CDR), which are those loops of a BCR or an antibody that directly interacting with the antigenic molecular structure. Mechanisms to change the specificity after V(D)J-recombination should avoid binding to self-structures. [1]

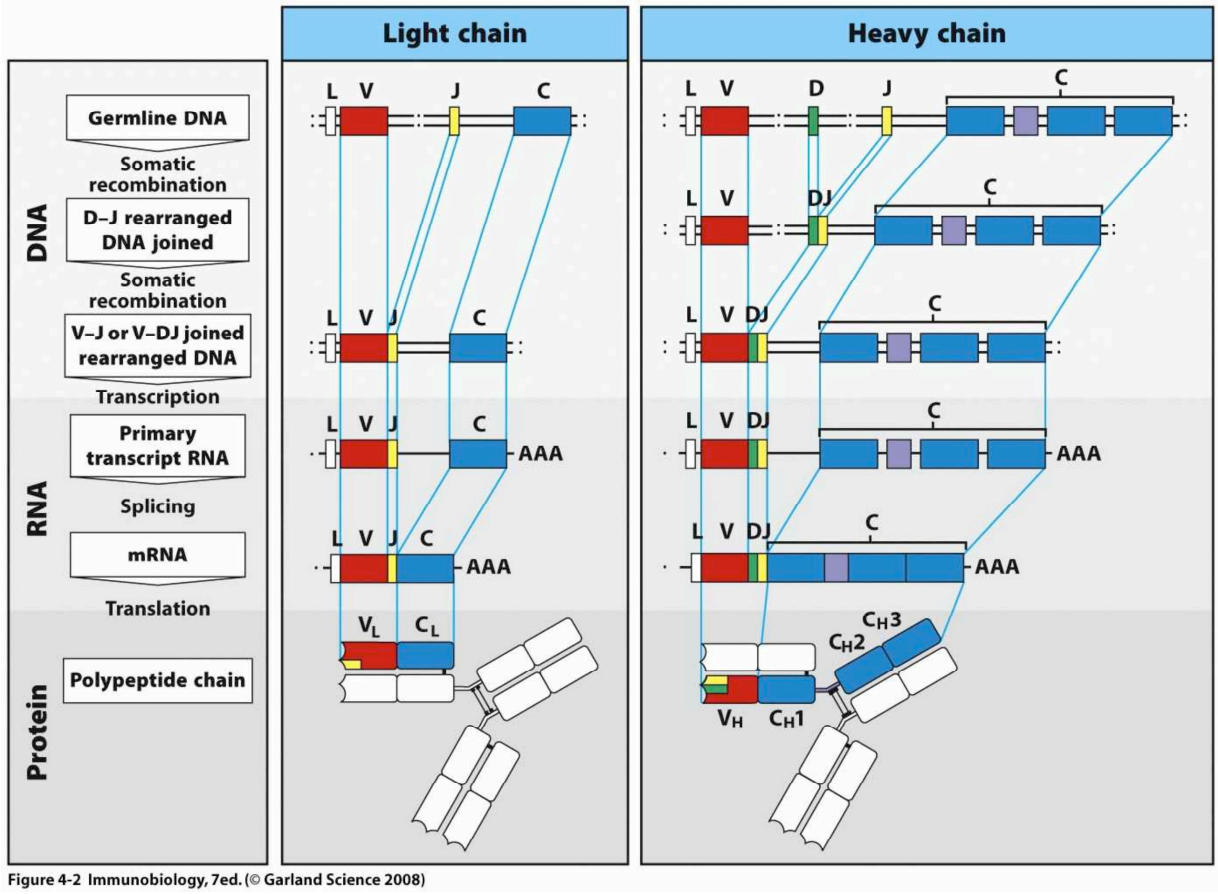

Figure 1 Somatic V(D)J-recombination of light and heavy chain of an antibody. [1] During V(D)J-recombination one V and one J DNA gene segment (light chain) and one V, D and J segment (heavy chain) are recombined at DNA level resulting in one recombined V-region gene domain. mRNA transcripts containing this V-region gene are spliced together with a constant region gene of a certain isotype. Polypeptide chains are assembled to form a functional antibody/BCR in the endoplasmatic reticulum.

1.1.5. Autoimmune Diseases - The Immune System is Imbalanced

Autoimmune diseases are disorders characterized by an adaptive immune response directed at self-antigens. Inflammations carried out by immune cells amplify the inflammatory signal. Compared to pathogen-caused inflammations self-antigens

cannot be eliminated easily because of their ubiquitous expression. Sustained inflammatory stimuli lead to chronic diseases with self-renewing feedback loops due to ongoing expression of the antigen maintaining disease symptoms.

Initially the recombination of gene segments followed by somatic hypermutations raises immune cells with potentially unlimited specificities, which also includes reactivities against self-structures. But to get rid of these poly- and self-reactive cells there are three major checkpoint mechanisms. [3]

Receptor editing in self-reactive precursor B cells in the bone marrow is used by combining and testing other V and/or J light chain gene segments.

Clonal deletion is defined as self antigen-induced death of autoreactive B-cells. Programmed cell death called apoptosis throughout the maturation of B-cell can be induced at certain developmental checkpoints, if cells display autoreactivity.

Anergy is another mechanism, where the proliferation of T- or B-cells is suppressed due to binding self-structures.

Autoimmune diseases only develop if one of these safeguards can be overcome due selective pressure when challenging pathogens, molecular mimicry of pathogenic structures with self-structures or injuries leading to accessibility of self-antigens that would not be encountered else by immune cells. Genetic contributions may be a major factor for the susceptibility to autoimmune diseases. Mutations in genes that are involved in the checkpoint safeguard machinery can be of critical importance. [1]

1.1.6. Rheumatoid Arthritis

Rheumatoid Arthritis is a chronic inflammatory disease that affects 0,5 – 3,0% of the Caucasian population. Patients suffer from inflamed joints due to an autoimmune response. Disease progression can lead to the erosion of bones and cartilages. Consequences are chronic pain and disability of joint function. Though a lot of investigations about the mechanisms underlying this disease have resulted in the development of new biologics, rheumatoid arthritis still remains a chronic disease with feasible relapse. [1, 4]

Multiple characteristics have been described for rheumatoid arthritis. Immune responses of T- and B-cells against certain autoantigens like collagen type II (CII), citrullinated peptides (CPP) or IgG's have been shown. The injection of pathogenic IgG antibodies against CII can enhance the development of arthritis in mice, claiming an important role of antibodies in this disease. [5, 6] Interestingly, antibodies against certain self-structures can be found in many but not every patient. [4]

1.2. Therapeutic Immunoglobulin G

1.2.1. IgG Structure

B-cell receptors (BCR) and antibodies consist of 2 heavy and 2 light chains that are each divided into variable and constant domains (**Figure 2**). Naive B-cells initially only express constant regions of heavy chains (C_H) of the C_μ and the C_∂ class (IgM and IgD). The C_H genes are encoded downstream of the rearranged VDJ-region segments. Thus, a naive B-cell that has undergone VDJ-recombination expresses one VDJ-region assembled to whether C_μ or C_∂ classes which leads to transmembrane IgM or IgD of same antigen specificity. If B-cells become stimulated by antigens they are able to undergo class switching, which allows them to link their VDJ-region to other C-region classes (**Figure 3**). Class switch recombination determines which Ig isotype an activated B-cell will secrete when it terminally differentiates into a plasma cell. [1]

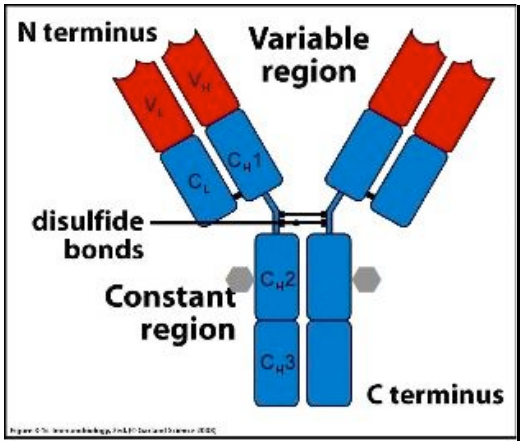

Figure 2 Antibody structure [1] (figure modified). An antibody consists of 2 identical long heavy chains containing 1 variable region domain (V_H) and 3 constant region (C_H) domains. Both chains are covalently linked via disulfide bonds (cystin bridges). The 2 shorter light chains bind via hydrogen bonds to the heavy chains and consist of 1 V_L and 1 C_L domain. Grey hexagons indicate glycosylation sites in the C_H2 domain of $C\gamma$ (IgG).

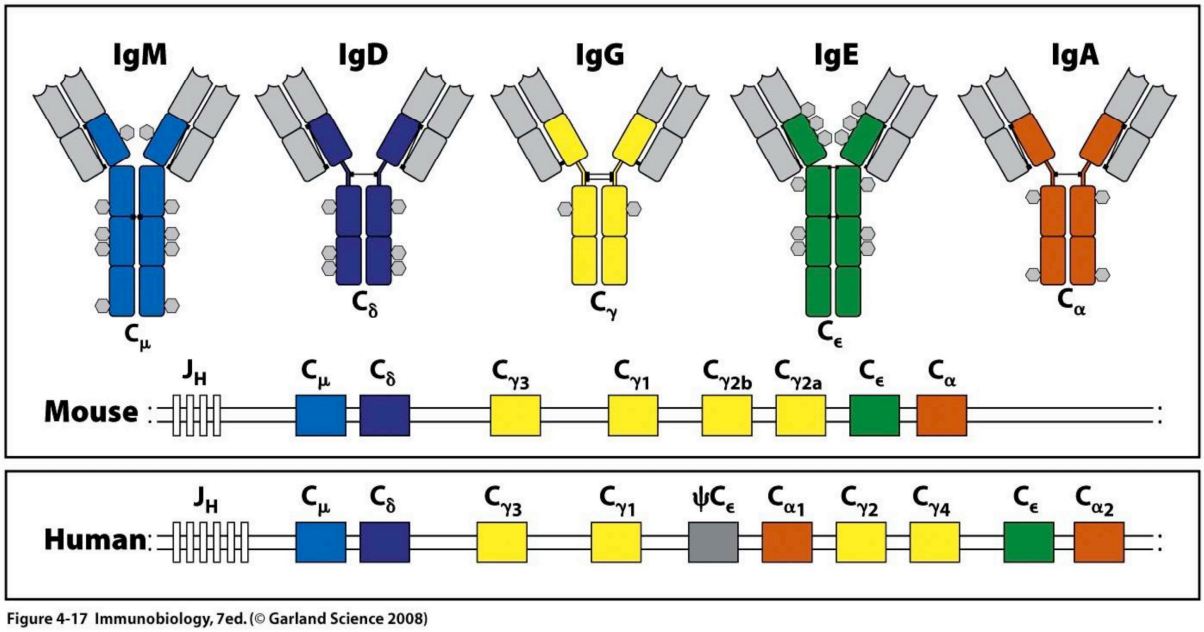

Figure 4-17 Immunobiology, 7ed. (© Garland Science 2008)

Figure 3 Immunoglobulin isotypes. [1] Activated B-cells undergo isotype switching, which means that upon cytokine stimulation the Ig heavy chain locus is recombined and the V-region is assembled with one C_H gene. Besides IgA (C_α), IgD (C_∂), IgE (C_ε) and IgM (C_μ) there are 4 isotype subclasses of IgG ($C\gamma$) genes. Grey hexagons indicate glycosylation sites. Mice and humans have homologues C_H genes.

Roughly 5% of serum antibodies are of IgM subclass. IgM antibodies usually appear at elevated levels at the beginning of an infection, because they are besides IgD the

first Ig-subclass to be expressed before B-cells undergo class switching. The most abundant isotype in the blood of vertebrates like humans and mice is IgG, which makes up to 85% of immunoglobulins in the serum. [2]

IgG antibodies are associated with pathogen clearance and toxin neutralization and are the primary mediators of protective humoral immunity and inflammation. [7] There are 4 different IgG subclasses in humans and mice. When a B-cell becomes activated and undergoes isotype switching it might rearrange with one of the 4 different IgG subclasses [1], which are all functionally different due to different binding properties to immune receptors that carry out certain effector functions. [8, 9] Mouse IgG2a and IgG2b are considered to have a high pathogenic potential to trigger inflammation in autoimmune diseases. [10]

Human	Mouse
IgG1	IgG2a
IgG2	IgG3
IgG3	IgG2b
IgG4	IgG1

Table 1 IgG subclass homologues in humans and in mice. Corresponding IgG subclasses between species are shown due to sequence homologies. Their abundance in blood serum and their effector functions though differ between species. Numbering of human and mouse IgG subclasses were done according to the blood serum levels. IgG1 is the most abundant isotype in the serum of one species.

1.2.2. Fc-Glycan Structure

Cleavage of antibodies with a proteolytic enzyme called papain leads to peptide fragments. Papain cleaves antibodies in the hinge region between the C_H1 and the C_H2 domain releasing the 2 antigen binding arms (Fab, fragment antigen binding) and the C_H effector region dimer (Fc, fragment crystallizable) (**Figure 2**). [1] In IgG antibodies this Fc-region contains the only glycosylation site at each heavy chain asparagine 297 (Asn297) having an N-linked biantennary oligosaccharide attached (**Figure 4**). [11, 12] These oligosaccharide glycans are sugar chains that point to each other influencing the conformation of the Fc effector region. IgG Fc-glycans are crucially necessary for their effector function. Specific cleavage of IgG Fc-glycans by

the bacterial enzyme endoglycosidase (EndoS) completely abrogates cytotoxicity of IgG antibodies. [13, 14, 15]

Different Fc-glycan structures have been associated with different effector functions of IgG antibodies. [14, 16] Even monoclonal antibodies produced by one hybridoma B-cell have different Fc-glycosylation pattern. There are 4 prominent Fc-glycan isoforms associated with effector functions. Short Fc-glycans have biantennary terminal N-acetylglucosamine, which can both be elongated with galactose and may terminate with sialic acid (**Figure 4**). [12, 17]

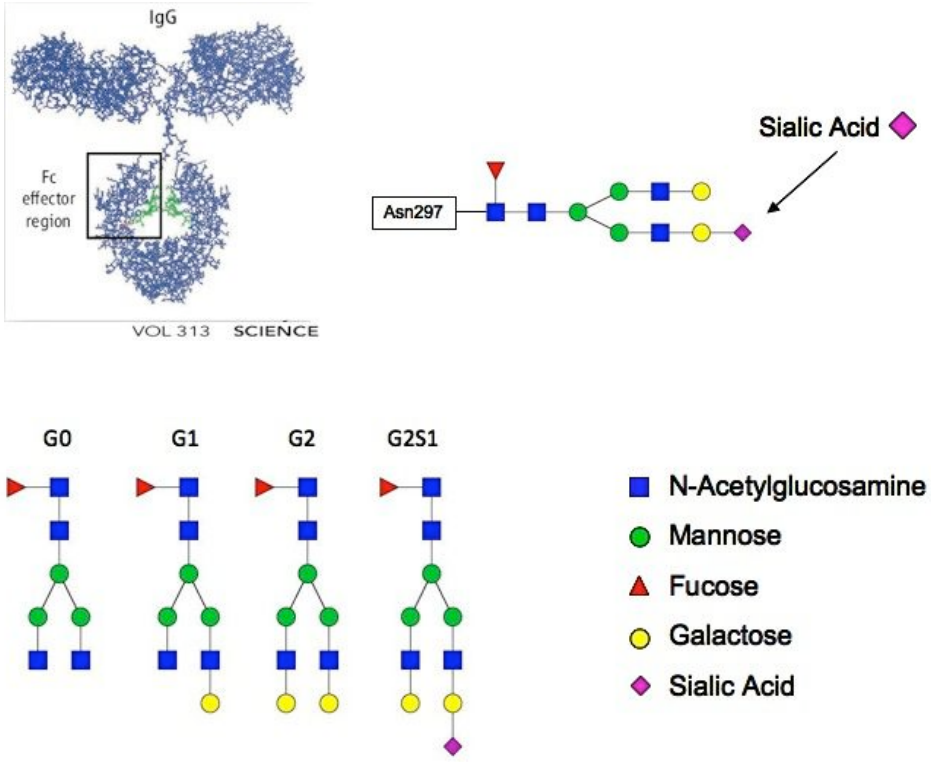

Figure 4 Fc-glycan structures. Primary N-acetylglucosamine is attached to the Asn297 of each heavy chain in the C_H2 domain. Depending on the terminal sugar this Fc-core oligosaccharide is associated with different effector functions. G0, G1 and G2 isoforms have no, 1 or 2 terminal galactoses. S1 and S2 isoforms have 1 or 2 terminal sialic acids. About 10% - 15% of serum IgG's from healthy donors harbour Fc-glycans with terminal sialic acid [12], which provide antiinflammatory properties to antibodies.

1.2.3. Fc-glycosylation in Autoimmune Diseases

It has already been reported in the 1980's that patients with rheumatoid arthritis have serum IgG antibodies with altered Fc-glycan structures compared to healthy individuals. Interestingly, whole blood serum IgG's in patients with chronic rheumatoid arthritis have shorter Fc-glycans with more than 60% of IgG's harbouring G0 glycans (**Figure 4**) and any glycans having terminal sialic acid. In healthy individuals up to 15% of serum IgG's have oligosaccharides with terminal sialic acid and most of the glycans are in the G1 or G2 isoform. Patients with rheumatoid arthritis that turned into remission back to a healthier state express again serum IgGs with predominantly longer Fc-glycan chains.

These findings were first indications that Fc-glycosylation patterns may have a strong influence on the function of IgG's in chronic inflammatory diseases. [16, 18]

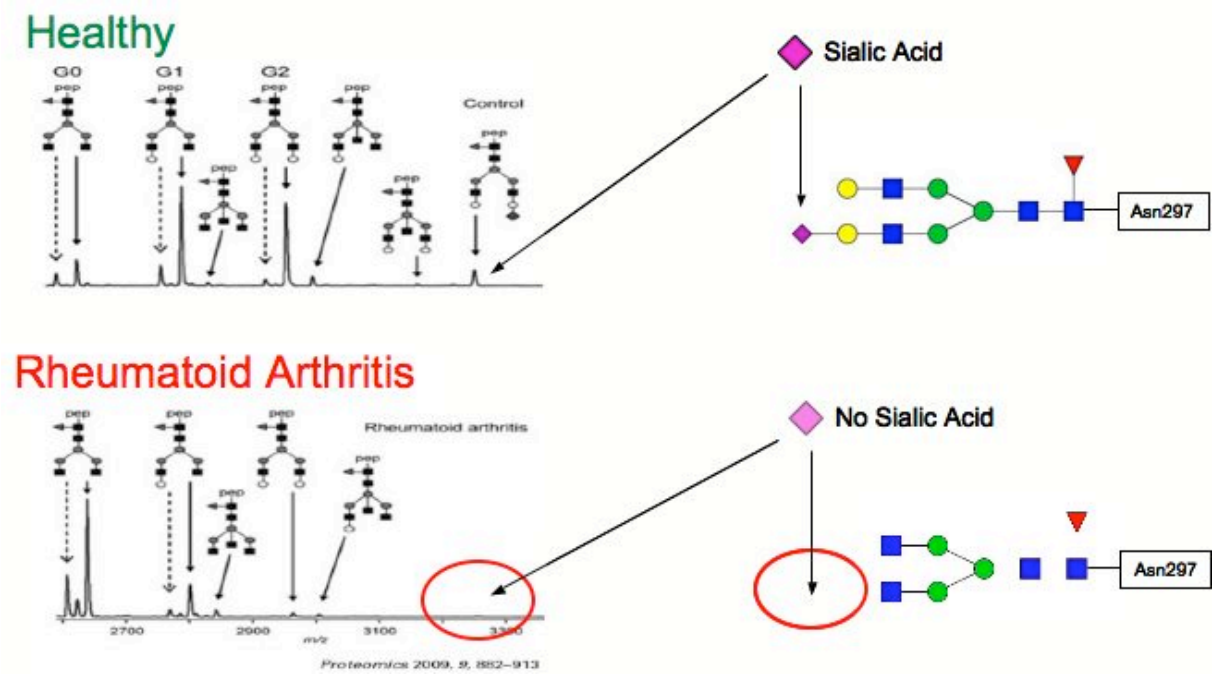

Figure 5 Mass spectroscopic (MALDI-TOF) analysis of IgG Fc-glycans. [12, 16] Each peak in the diagram represents the amount of Fc-glycans of a certain isoform. In rheumatoid arthritis there is a prominent peak at about 2650 m/z, which means compared to the other peaks, that most glycans accelerated in the mass spectroscopic analysis are in the G0 glycoform having no galactose and no terminal sialic acid. Only healthy individuals display Fc-glycans with terminal sialic acid (about 3250m/z), which therefore became attributed to have antiinflammatory effector properties.

1.2.4. Intravenous Immunoglobulin G (IVIG) Therapy

Intravenously injected immunoglobulin G (IVIG) is an approved therapeutic containing pooled serum IgG of all subclasses from up to 10 000 healthy donors. IVIG has turned out to be a potent tool not only to treat immunodeficient patients but also for patients with autoimmune diseases. High dose IVIG shift the antibody response in the blood towards a healthy state but mechanisms of action have still not been solved completely.

In 2008 Anthony *et al.* recapitulated the antiinflammatory activity of IVIG with sialylated Fc portions.[19] IgG Fc dimers without Fab fragments could provide the same benefit as full-length IVIG antibodies and this effect was only due to terminal sialic acid on the Fc-glycan. Fc-fragments without terminal sialic acid showed no beneficial efficacy in autoimmune disease models.[19, 20]

Rheumatoid arthritis is an autoimmune disorder where alterations in the Fc-glycosylation patterns of IgG antibodies can be observed (**Figure 5**).[21] Several cases of IVIG therapy provided benefit to the patients with rheumatoid arthritis, probably due to a shift of the Fc-glycan isotype distribution of serum IgGs back to a healthy glycosylation state with terminal sialic acid. However, studies have proved therapeutic effects of IVIG in rheumatoid arthritis treatment, but detailed models of the efficacy are lacking.

1.2.5. Contraindications of IVIG

Thus Fc sialic acid is a key sugar that provides IgG antibodies with antiinflammatory effects and production of antibodies without sialylated Fc-glycans may provide a shift from a healthy steady state to inflammation.

But there are several disadvantages in the treatment of patients with IVIG. It has to be given in very high doses of around 2g/kg per month to eclipse the intrinsic IgG effects. Due to the production procedure IVIG is a very cost intensive therapy. Frequently side effects like headache and fatigue are reported. Beyond, IVIG provides a systemic effect and even the transfer of allergies due to IgG's from donors

that target allergens have been reported. So scientists strive for more specific options exploiting the beneficial effects of IVIG. [7, 19, 22, 23, 24]

1.2.6. How IVIG Work

IgG antibodies can bind components of the complement system, but they also interact with immune cells via Fcγ-receptors. There are several different activating Fcγ-receptors, which upon crosslinking after binding to the Fc-portions of IgG's trigger proinflammatory signalling pathways. This leads to the expression of proinflammatory cytokines such as TNF-α, IL-1 and IL17, a prominent cytokine in autoimmune diseases. There is only one inhibitory Fcγ-receptor on immune cells, the Fcγ-receptor II B (FcγRIIB). [25] Activation of the FcγRIIB upon binding of IgGs starts a signalling cascade that leads to a feedback loop with enhanced expression of FcγRIIB. The ratio of activating to inhibitory signalling decreases and the threshold to trigger immune responses rises. This results in the protection of self-structures from further threat by inflammation.

IgG antibody subclasses show different affinities to activating and inhibitory Fcγ-receptors with mouse IgG1 showing the lowest activating to inhibiting ratio. Sialylation of IgG antibodies reduced their cytotoxicity due to lowered affinities of IgGs to Fcγ-receptors. This mechanism is supposed to ensure steady-state serum IgGs to maintain calm. But when challenged with pathogenic antigens the antigen-specific IgG's can be switched to a population of reduced sialic acid to provide inflammatory effector functions for proper pathogen clearance. [9, 20]

It has been shown that the antiinflammatory effect of IVIG can be translated by splenic marginal zone macrophages via a C-type lectin receptor called SIGN-R1 (specific ICAM-3 grabbin non-integrin related 1) in mice. [22] This receptor is only expressed on splenic marginal zone macrophages and is crucial for the antiinflammatory function of IVIG because upon binding of sialylated Fc's, marginal zone macrophages induce upregulation of the inhibitory FcγRIIB on effector macrophages, so the ratio of inhibitory to activating Fcγ-receptors increases. The human homologue DC-SIGN is not expressed on macrophages but dentritic cells.

Therefore results concerning the activity of this receptor must be translated to humans with care. [26]

There are only few publications suggesting any functional pathway on how sialylated antibodies act at sites of inflammation except of studies showing that sialylated IgGs have reduced affinities to Fcγ-receptors. The influence of sialylated IgG's on other immune cells like T-cells is currently subject of investigations. [27] [28]

Recent findings have described the interference of IVIG in antigen presentation and therefore indirectly influencing the activation of T- cells. [28] IVIG-Fc binding reduced the ability of antigen-presenting cells to present antigens via MHC class II to $CD4^+$ T-cells, which did not become activated. This effect of IVIG was shown to be independent of the inhibitory FcγRIIB. Binding of IVIG-Fc to antigen-presenting cells occurred via activating Fcγ-receptors.

1.2.7. Sialylated Antigen-specific Antibodies

IVIG has a systemic shut down effect on the immune system, but if there is a way to direct this anti-inflammatory property of sialylated IgGs to interfere antigen-specifically in the inflammatory response this must lead to the antigen-specific shut down of inflammatory signals. Inhibitory effector functions of sialylated Fc's combined with antigen-specificity of the Fab fragment may lead to a precise immune regulation without systemic side effects.

Anti-CII antibodies have been shown to bind CII in mouse joints in vivo and to induce severe arthritis, [5, 6, 29] therefore suggesting that sialylated anti-CII IgGs antigen-specifically target CII in the joints. Interference of sialylated CII-specific IgGs in antigen presentation and therefore in T-cell activation is one proposed mechanism leading to inhibitory effects in the development of arthritis.

However, we did not know if any other receptor (probably related to SIGN-R1) than the inhibitory FcγRIIB expressed on immune cells leads to immunosuppressive signalling or if even activating Fcγ-receptors of antigen-presenting cells translate antibody binding into antiinflammatory responses, like suggested by Aubin et al. [28]

The FcγRIIB is crucial in counterbalancing autoimmunogenic stimuli. [30] Thus, in FcγRIIB$^{-/-}$ knock out mice beneficial effects of sialylated CII-specific antibodies must elicit other regulatory mechanisms.

1.2.8. Treat Autoimmune Disease With Sialylated IgG's

To examine if we can use the antiinflammatory effect of sialylated IgG's in an antigen-specific way, we chose an autoimmune disease model with a defined immune response. A mouse model for rheumatoid arthritis is chicken collagen-induced arthritis (CIA), where mice are immunized with heterologues chicken collagen type II (CII). The initiation of an immune response harbours the risk of a cross-reaction with self-CII, which is a component of the synovial fluid in joints. If adjuvant stimulation is strong enough, tolerance of self-CII can be broken and an excessive immune response with high potential for cross-reaction with self establishes.

In CIA there is a defined antigen (CII) that can be targeted by CII-specific IgG antibodies. Usually these antibodies promote the disease. [6] So we glycosylated the Fc-glycan of the anti-CII IgG's by attaching galactose and terminal sialic acid to the oligosaccharide chain. These sialylated antibodies targeted chicken and mouse CII to direct their antiinflammatory effects to foreign and self CII.

The idea was to prevent mice from the development of arthritis due to inhibiting an immune reaction against CII by applying therapeutic antibodies (sialylated anti-CII IgG1) before the immunization with chicken CII. We hypothesized that upon the trigger of an immune response against CII, the antigen is already protected by sialylated IgG's that reflect a normal steady-state sialylation, which prevents the mice from the development of inflammatory immune responses against chicken and self-CII and thereby of arthritis.

IgG1 antibodies were used, because it has been shown that they have less cytotoxic activity compared to IgG2a or IgG2b subclasses. [9, 10] Additionally, we investigated the

effect of sialylated IgG1 antibodies in FcγRIIB$^{-/-}$ mice to analyze the role of the inhibitory FcγRIIB.

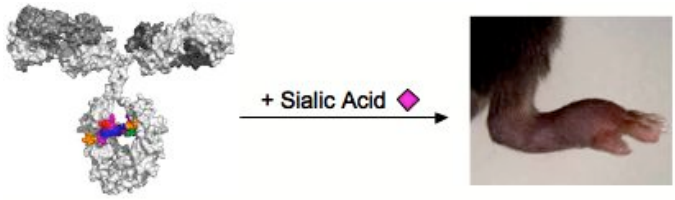

Figure 6 Therapeutic anti-CII IgG antibodies are sialylated to induce tolerance to chicken and self-CII and to inhibit joint swelling.

1.2.9. Glycosylation of Therapeutic Antibodies for Clinical Applications

Since the 1990's therapeutic IgG antibodies have become the fastest growing class of human therapeutics and the second largest class of drugs after vaccines. IgG antibodies for passive immunization to clear pathogens as well as for the treatment of chronic inflammatory diseases or tumors have been approved to the market. Recent findings have suggested the IgG Fc-glycan to have strong modulating effects. E.g. if core fucose is removed, antibodies display increased cytotoxic effector functions. [31]

Sialylation of recombinant proteins has been carried out by industry to increase the half-live of biologics, but recent findings propose that sialylation of IgG Fc-glycans reduces their ability to engage Fcγ-receptors and even provide tolerance to the antigenic structures. Fc-glycans with terminal sialic acid have therefore become a target of autoimmune disease research. Self-antigen-specific sialylated IgG with antiinflammatory activity are a potent future tool to inhibit inflammatory diseases like rheumatoid arthritis. [11, 14, 15, 32, 33]

2. Aim

Therapeutic antibodies have become an increasingly emerging market. Antibody based biologics have been developed to targed and clear pathogen-associated structures and malignant cells as well as inflammatory cytokines in chronic inflammatory disease. Recent findings suggested that the effector function of IgG antibodies can be modified upon structural changes of their Fc-glycan. Terminal sialic acid on this Fc-glycan gives IgG antibodies antiinflammatory properties providing switch mechanisms between a healthy steady-state with tolerance against specific antigens and activating effector functions.

We used the chicken collagen-induced arthritis mouse model, which reflects a rheumatoid arthritis-like phenotype, as autoimmune disease model with defined immune response against one self-antigen (collagen type II).

The aim of this project was to newly design self-antigen-specific IgG antibodies with antiinflammatory activity due to Fc-sialylation to prevent the development of arthritis in mice. Our goal was to develop a novel therapeutic approach to treat autoimmune diseases - not by blocking proinflammatory cytokines - but to directly target the immunogenic self-antigen (collagen type II) with sialylated IgG antibodies to enhance tolerance. Antigen-specific application should lead to individualized therapies avoiding the systemic and negative effects of IVIG and could be administered at much lower dose to prevent from side effects.

3. Materials and Methods

3.1. Expression Vector Cloning

3.1.1. Anti-Collagen Type II Antibody Sequences

We used sequences of antibodies specific for collagen type II (CII) of different species that have been described in the literature. However, articles describing nucleotide sequences of the variable region (V-region) of anti-CII IgG's usually focus on the complementary determining region (CDR) sequences, which are of main interest when studying the interaction of antibodies with antigens. To obtain the full-length nucleotide sequences of the V(D)J-regions of IgH (heavy) and IgL (light) chain genes, which were necessary for cloning, we completed V(D)J-region sequences of anti-CII antibodies [34, 35, 36] (**Table 2**) with their corresponding germ line gene sequences that were determined by IgBLAST comparison with GenBank database (http://www.ncbi.nlm.nih.gov/igblast/) and sequences of the IMGT database (http://imgt.cines.fr) [36] (**Table 3**). Additionally we corrected mismatches between the anti-CII IgH and IgL chain sequences and their germ line genes that appeared upstream of the CDR1. Sequencing errors could have been the reason for these mismatches. In case of doubt we used the germ line sequence.

If IgH or IgL chains were described in part as amino acid sequence in the literature, [36] the triplet codon of the corresponding germ line gene was corrected by single base exchange according to the germ line gene segment. To ensure segregation of the antibody chains we added a leader sequence (GenBank accession number DQ407610) also containing an AgeI restriction site for cloning. At the end of the heavy chain VDJ DNA sequence a SalI restriction site was introduced to later

combine the VDJ sequence with the constant murine IgH chain in frame. Accordingly at the end of the light chain VJ DNA sequence a BsiWI restriction site was introduced to later combine the VJ sequence with the constant murine Igκ sequence in frame.

The final anti-CII antibody sequences (**Table 4**) of the IgH and IgL chain V-regions were ordered at MrGene (https://mrgene.com).

Antibody	IgH	IgL	Mouse Strain
aCII 1-5 [35]	U69538	U69539	DBA/1J
M2139 [36]	Z72462	Z72463	DBA/1
ACC1 [36]	EU159566	EU159567	B10.RIII.Cia5
CIIC1-GG [36]	Z72441	Z72442	DBA/1
CIIC1-E [36]	Z72441	Z72442	DBA/1

Table 2 GenBank accession numbers. Nucleotide sequences of the V(D)J-regions of heavy and light chains of anti-mouse CII antibodies used as templates for assembling our antibody sequences.

Antibody	Ig heavy chain	Orig. Isotype	V-gene	D-gene	J-gene	Ig kappa chain	V-gene	J-gene
aCII 1-5 [35]		IgG2a	J558.45	DFL16.1	JH2		12-44	JK1
M2139 [36]		IgG2b	J558.45	DSP2.8	JH2		21-1	JK2
ACC1 [36]		IgG2c	J606.4.82	DST4.2	JH2		21-2	JK2
CIIC1-GG [36]		IgG2a	J558.40	DST4.2	JH3		21-5	JK2
CIIC1-E [36]		IgG2a	J558.40	DST4.2	JH3		21-5	JK2

Table 3 Corresponding germ line VDJ IgH gene segments and VJ IgL kappa gene segments used to fill up incomplete sequences. Mutations far upstream of the CDR1 region have been reverted according to their germ line V-genes. Germ line gene segments were determined by IgBLAST and IMGT database.

3.1.2. Expression Vector

To co-express the IgH and the IgL chain of each antibody in cell culture we used expression vectors for murine IgG1 heavy chains (mIgG1) and murine kappa light chains (Igκ). [3, 37, 38] The vectors (**Figure 7**; GenBank accession number DQ407610) were a kind gift of Dr. Hedda Wardemann (Max-Planck-Institute for Infection Biology, Berlin).

Upstream of the mIgG1 and the mIgκ sequences there is a multiple cloning sites (MCS) containing AgeI and SalI (IgG1) and AgeI and BsiWI (Igκ) restriction sites to insert the V(D)J region sequences. The human cytomegalovirus (HCMV) promoter provides a strong transcriptional activity. Selection occurs via an ampicillin resistance cassette.

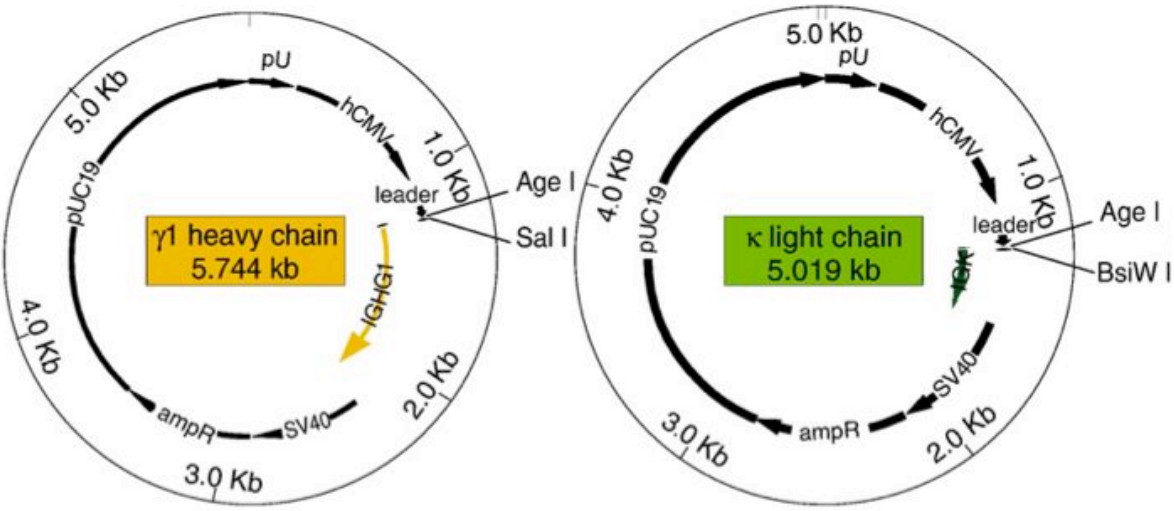

Figure 7 Expression vector maps.[38] The human cytomegalovirus promoter is followed by a leader sequence, a MCS for inserting the V gene sequences and an IgG1 (γ1 heavy chain) or an Igκ (κ light chain) constant region. The eukaryotic expression vectors are promoted in bacterial clones based on ampicillin resistance.

3.1.3. Restriction Digest

The VDJ region sequences of the IgH chains were cut out of the vector backbone delivered by MrGene with AgeI and SalI restriction enzymes. The VJ regions of the IgL chains were cut with AgeI and BsiWI (all enzymes by NEB – New England Biolabs). IgG1 heavy chain and Igκ light chain expression vectors were digested analogous to create sticky DNA ends, where the V(D)J sequences could be inserted [37, 38] The reaction conditions were set up according to the manufacturers instructions (NEB). Just in brief, 3μg DNA were digested with 3U restriction enzyme in 30μl total reaction volume containing 3μl of the proper 10x enzyme buffer (AgeI and SalI (IgH) in NEB-4 buffer, AgeI and BsiWI (IgL) in NEB-1 buffer). IgH (AgeI, SalI) sequences

were double digested at 37°C for 1h. Igκ sequences were digested sequentially. After AgeI digest for 1h at 37°C, 3U BsiWI were added and temperature was raised to 55°C for 1h.

3.1.4. Fragment Purification

Prior to ligation the digested V-region fragments were seperated from the vector backbones on a 0,9% agarose gel (1xTAE buffer (Tris-acetate-EDTA), 1µl ethidium bromide per 20ml agarose gel; Orange G loading dye) for 30 minutes at 140V. Fragments were cut out of the gel with a scalpel and purified with NucleoSpin® Extract II kit (Macherey-Nagel) according to manufacturers instructions.

3.1.5. Ligation

Reaction volumes for ligation of the V-region fragments into expression vector backbones were calculated with GENtle software. The total reaction volume was 10µl containing 2µl 5x T4 DNA-Ligase reaction buffer (Invitrogen). 100ng DNA with a molar insert to vector ratio of 3:1 was ligated by 1U of T4 DNA-Ligase (Invitrogen) for 20 minutes at room temperature.

3.1.6. Transformation

Competent E. coli DH10B bacteria (Clontech) were thawed on ice and incubated with 100ng ligated DNA for 5 minutes on ice. The transfer of the expression vector into the bacterial cells – called transformation – was carried out for 30 seconds on 42°C. After 1 minute on ice 250µl pre-warmed SOC-medium were added and transformed bacteria recovered for 30 minutes shaking at 37°C. 100µl transformed bacteria were plated onto lysogeny broth (LB) ampicillin (100µg/ml) plates and incubated over night at 37°C.

The antibiotic ampicillin was used to select for ampicillin resistant clones carrying our expression plasmid with an ampicillin resistance cassette, which expresses the enzyme beta-lactamase to cleave cell wall synthesis inhibiting ampicillin in the

medium. Only bacteria expressing beta-lactamase could proliferate to from colonies in the presence of ampicillin.

3.1.7. Plasmid Preparation

Colony forming units (CFU) of single bacterial clones carrying the transformed plasmid were picked and grown in 4ml liquid LB-medium over night shaking at 37°C. Preparation of plasmids (NucleoSpin® Plasmid, NucleoBond® Xtra Maxi Plus, all by Macherey-Nagel) was done due to instruction guidelines. Just in brief, after centrifugation the cell pellets were resuspended and cells were lysed in an SDS/alkaline buffer to set the plasmids free. Genomic DNA, which is attached to the bacterial cell membrane, is washed away after neutralization. Clarified lysates are transferred to a silica membrane where plasmid DNA keeps bound throughout several washing steps until elution with 50μl elution buffer (5mM Tris/HCl, pH8.5).

3.1.8. Sequencing

To screen V-region inserts of the IgG1 and Igκ chains for correct insertion into the vector and for mutations we delivered the final constructs containing the aCII-antibody IgH and IgL chains to MWG (http://www.eurofinsdna.com/de/) for sequencing. Primers [37] were binding upstream of the insert in the leader sequence (Ab-sense) of the aCII-antibody chains and in the respective murine IgG and murine Igκ C-region.

Insert check	Primer name	5'-3' sequence
	5′ Absense [37]	GCTTCGTTAGAACGCGGCTAC
	3′ IgG (internal) [37]	GTTCGGGGAAGTAGTCCTTGAC
	3′ Cκ 494 [37]	GTGCTGTCCTTGCTGTCCTGCT

Table 4 Primer sequences for the insert screen. 5' Absense binds in the leader. 3' primers bind in the C-region of IgH (IgG internal) and IgL (Cκ 494). Sequencing products reveal mutations and correct insertion of the V(D)J-region fragment.

3.2. Antibody Expression

3.2.1. Cell Culture

For the expression of the anti-CII mouse antibodies we used an immortalized human cell line. Those human embryonic kidney 293T (HEK293T) cells were cultured on 15cm tissue plates (Greiner) in 25ml Dulbeccos modified eagle medium (DMEM + GlutaMax™-I; Gibco) containing 10% fetal calf serum (FCS) and 10ml/L penicillin/streptomycin (by PAA) and incubated at 37°C and 5% CO_2. Optimal growing condition could be achieved when passaging the cells twice a week at a confluency of 80%. The cells were trypsinised with 3ml of trypsin-EDTA (PAA) after removal of the medium and washing with 15ml 1xPBS and distributed to new culture dishes at a ratio of 1:3 – 1:10.

3.2.2. Transfection

Best transfection efficiency could be achieved at a cell confluency of about 70% at the time of transfection. Cells should be in exponential growth phase (log phase) with least contact growth inhibition.

Cell culture dishes were washed with 1xPBS before adding transfection medium containing 22ml DMEM per plate with 2,5% primatone in PBS (Kerry Primatone RL/UF 5X59057) cell culture additives and 10ml/L penicillin/streptomycin (PAA) but no FCS to avoid IgG contamination. 10μg heavy chain and 10μg light chain plasmid DNA (per plate) are vortexed for 30s with 0,6μg/μl of branched polyethylenimine (PEI, Sigma 408727) in a final volume of 3,000μl PBS. Positively charged PEI forms complexes with negatively charged DNA, which can be taken up by the cells when the transfection mix is carefully added to the transfection medium on the cell culture dish. The cell medium supernatant was harvested after 4-6 days of incubation at 37°C and 5% CO_2.

3.2.3. Antibody Purification

Harvested supernatant containing the secreted antibodies was centrifuged 10min at 4000rpm to remove cell contamination followed by sterile filtration in a Stericup® (Millipore). Clear sterile supernatant was run through a Protein G Sepharose® (GE Healthcare) column. Binding to protein-G in the column retained the antibodies. Elution was performed by adding 5x the column bed volume of 0,1 glycin buffer (pH3). The fractions were collected and neutralized with 1/20 of the elution volume of 1M Tris/HCl buffer (pH 8,5). Antibody concentration, which was usually high in fractions 2 and 3, was determined by photometric analysis at a wavelength of 280nm (NanoDrop).

Before storage at -80°C the antibody solution was dialysed over night against 1xPBS.

3.2.4. SDS-PAGE

The expression of both, antibody heavy and light chain in the HEK293T cells was verified via denaturating polyacrylamid gel electrophoresis (PAGE). 10μg of each purified antibody sample mixed with protein loading buffer was heated for 10min at 94°C. Loading buffer contained sodium dodecyl sulfate (SDS), which is an amphiphilic detergent where the non-polar carbohydrate chain (C-12) wraps around the heat denatured protein backbone. The anionic sulfate head of the molecule provides charge to the SDS-protein complex. The larger a protein the more SDS molecules can attach, which provides every protein chain with the same mass to charge ratio of 1,4μg SDS per 1μg protein. Protein migration in an electric field occurs due to size in a polyacrylamid gel. The intrinsic charge of the protein becomes negligible. Loading buffer (0,25M Tris-HCl) additionally contains glycerin to give the samples more density for optimal loading conditions. β-mercaptoethanol reduces disulfide bridges for complete denaturation of the protein. Bromphenol blue is used as a tracking dye.

A protein ladder (Kaleidoscope; BioRad) with recombinant prokaryotic proteins of known size was used as molecular weight marker.

10% AA (acrylamid/bisacrylamid) resolving gel was made with 5,9ml H_2O, 5,0ml acryl-bisacrylamide mix (30% AA), 3,8ml 1,5M Tris (pH 8,8) and 150μl SDS. 150μl

ammonium persulfate (10% APS) initiates gel formation when finally 6µl of TEMED are added to start polymerization.

5% AA stacking gel was formed by mixing 5,5ml H_2O, 1,3ml AA (30%), 1ml 1,5M Tris (pH 6,8) and 80µl SDS (10%). Polymerization was started with 80µl APS (10%) and 8µl TEMED.

The gel was run at 140V for 3h.

3.2.5. Enzymatic Glycosylation

Enzymatic *in vitro* glycosylation was performed in a two-step procedure by first galactosylating and second sialylating the antibodies [19]. Buffer exchange of 5mg of one antibody at a concentration of 1mg/ml was carried out in a 5ml Amicon® Ultra-4 Centrifugal Filter Units (50kDa size exclusion; Millipore) according to manufacturer's instruction. MOPS buffer (50mM, pH 7,2) was carefully(!) added drop wise after centrifugation steps until no PBS buffer was left. Phosphate ions of PBS would precipitate brown after addition of mangan.

1M $MnCl_2$ (in H_2O) was added to a final concentration of 20mM. Mangan is a necessary cofactor of the β-1,4-galactosyltransferase [5mU/µl] (Sigma 48279), which was added to a final concentration of 75mU/ml with 5,3mg/ml UDP-Gal [0,25mg/µl]. The galactosylation was run for 48h at 37°C shaking. White precipitates, which indicated enzymatic activity, were removed via centrifugation and reaction buffer was exchanged in an Amicon® Centrifugation Filter Unit (Millipore) to 50mM MES (pH6) containing 10mM of 1M $MnCl_2$ and 0,5mg CMP-Sial [25µg/µl]. After addition of 50mU of β-1,6-sialyltransferase [1,43mU/µl] (St6Gal1, Calbiochem 566223), sialylation was carried out for 48h at 37°C shaking. Finally antibody solutions were washed with 100mM $NaCl_2$ to remove any mangan and buffered in 1xPBS. Antibody concentration after galactosylation and sialylation were determined by photometric measurement at 280nm (NanoDrop).

Efficiency of enzymatic glycosylation and antibody Fc-N-glycosylation pattern were analysed by MALDI-TOF. Reactivity pattern of antibodies were verified via ELISA.

3.2.6. MALDI-TOF Analysis

MALDI-TOF mass spectrometry was used to resolve Fc-oligosaccharide structures. IgG antibody samples were digested with endoglycosidase S (EndoS) purified from *Streptococcus pyogenes*. EndoS specifically cleaves the N-linked oligosaccharide at the Fc-fragment exclusively from IgG antibodies between the first and second N-acetylglucosamine (GlcNAc). Cleaved oligosaccharides were purified by solid phase extraction using reversed-phase C18 and graphitized carbon columns (Alltech, Deerfield, IL). Oligosaccharide samples were permethylated according to standard protocols and further investigated by MALDI-TOF mass spectrometry. Spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser. Calibration was performed on a glucose ladder and DHB was used as a matrix. Spectra were recorded in reflector positive ionization mode and mass spectra from 3000 laser shots were accumulated.

3.2.7. Enzyme-linked Immunosorbant Assay (ELISA)

The reactivity pattern of the anti-CII antibodies was determined via **reactivity ELISA**. Collagen type II [2mg/ml solubilized in 0,05M acetic acid over night at 4°C (better would be 0,01M acetic acid)] of bovine, chicken, human and mouse origin was coated at 2μg/ml (gelatine, dsDNA, ssDNA, insulin and LPS at 1μg/ml) in 100μl per well on a high binding microtiter 96-well plate over night at 4°C. After 3x washing steps with ddH_2O, free antigen binding sites were blocked for 1h at room temperature with 200μl/well with blocking solution (50mM Tris pH8, 1mM EDTA, 140mM $NaCl_2$, 0,02% Tween®20, 1% w/v BSA). 100μl of anti-CII antibody samples [1-10μg/ml] and 3-7 serial 1:3 dilutions of these samples in blocking solution were loaded to the microtiter plate after removing blocking solution. Binding of the antibodies to the CII occurred during 1h of incubation at room temperature and after another 3 washing steps with ddH_2O. Another 3 washing steps were followed by 1h incubation at room temperature with a horseradish peroxidase (HRP) coupled goat antibody against the murine anti-CII-antibody isotype. (IgG1, IgG2a, IgG2b, IgG3, Ig-Fc, IgM or IgA) 3x washing was finally followed by a 1-10min incubation of the samples with 100μl TMB (BD). Intensity of the TMB colorant that is cleaved by the HRP of the secondary

antibody is a direct measurement of the amount of primary antibody bound to CII. The reaction was stopped by adding 100µl 1M H_2SO_4. Analysis of the colour intensity was done with a microplate reader at 450nm.

To analyse different isotypes of serum immunoglobulins from arthritic and non-arthritic mice of different treatment groups via **serum ELISA**, the same ELISA setup was used as discribed above. Coating half of the wells of one microtiter plate with mCII and the other half with chCII. Serum (1:200 in PBS) was added and bound antibodies were detected on the whole plate with a secondary antibody (HRP-coupled) against one specific isotype (e.g. IgG1, IgM, ...). Analysis of the isotype distribution in the different groups was performed as described above according to the colorant intensity in each sample.

3.3. Collagen-Induced Arthritis Mouse Model

3.3.1. Mice

The induction of autoimmune diseases like collagen-induced arthritis is commonly elicited in genetically susceptible mouse strains like DBA/1 mice. Susceptibility is restricted by the class II molecules of the MHC haplotype H-2^q and H-2^r. [39, 40] Nevertheless C57BL/6 (Black 6) mice, which were usually regarded as being resistant to collagen-induced arthritis, became a target of studying autoimmune diseases, because most genetically modified mouse strains are on a C57BL/6 background (H-2^b) and recent publications have provided protocols for reproducibly induce arthritis in H-2^b haplotypes. [40, 41]

It has been shown that FcγRIIB$^{-/-}$ knock-out mice on C57BL/6 background are more susceptible to autoimmune disease. [30] Genotypes were determined by PCR on tail DNA as described previously. [10] Exclusively male FcγRIIB$^{-/-}$ mice bred in the DRFZ mouse facility Marienfelde were used for the treatment of CIA. Mice were bred and maintained in accordance with federal laws and institutional guidelines.

3.3.2. Reagent Setup

Bovine collagen type II (bCII) is commonly used to induce CIA in susceptible mouse strains. To reproducibly induce CIA in more autoimmune resistant mice harbouring the H-2^b MHC haplotype, we used chicken collagen type II (chCII, Sigma), which was dissolved over night at 4°C to 2mg/ml in 0,05M acetic acid.

1vial (100mg) grinded *Mycobacterium tuberculosis* (H37Ra, Difco Laboratories) was added to 25ml Complete Freund's adjuvants (CFA, Sigma) to increase H37Ra content from 1mg/ml to 5mg/ml. Effective immunization with sufficient amount of *M. tuberculosis* as immunogenic stimulus is necessary to overcome tolerance in H-2^b haplotypes. [40]

3.3.3. Collagen-Induced Arthritis

To finally have 100*µ*l of CFA/chCII immunization mixture per mouse 100*µ*l CFA (5mg/ml H37Ra) was vortexed with an equal volume (100*µ*l) chCII [2mg/ml] in a 15ml tube (Greiner) and about 800*µ*l of this immunization mixture were aliquoted to 1,5ml tubes (Eppendorf). chCII was thaw and handled on ice to avoid denaturation. For application it is critical that the emulsion is thick enough not to drip out of the tube when inverted [41], so we homogenized the immunization emulsion in the 1,5ml tubes with 2x4 short burst using a sonicator at 70% intensity to avoid heating and denaturation of the CII. The emulsion was collected with a 19G needle in a 1ml syringe. To avoid bubbles the syringes were inverted and tipped strongly at the bench edge to collect bubbles at the top, which were then pumped out of the syringe.

2x50*µ*l of the immunization emulsion were injected per mouse right and left up the base of the tail sub cutanously. [41] A booster injection was given 14 or 21 days analogue to the immunization but at more anterior site. C57Bl/6 mice were immunized a second time with an emulsion containing CFA (5mg/ml H37Ra) [39]. RIIB$^{-/-}$ mice were boosted with an emulsion containing IFA (Incomplete Freund's Adjuvant, Sigma) instead of CFA.

3.3.4. Clinical Assessment of Arthritis

Clinical assessment of arthritic paws was done according to standard protocols. [40, 41] Limb swelling of all animals was monitored 3x a week. The clinical score was assessed by scoring each limb of a mouse from 0 (healthy) to 3 (severe swelling of digits, paw and ankle). The maximum score per mouse is 12. The mean clinical score was calculated by including all animals – even healthy ones - of one group. Arthritis incidence was considered when at least on limb of a mouse was clinically scored 1.

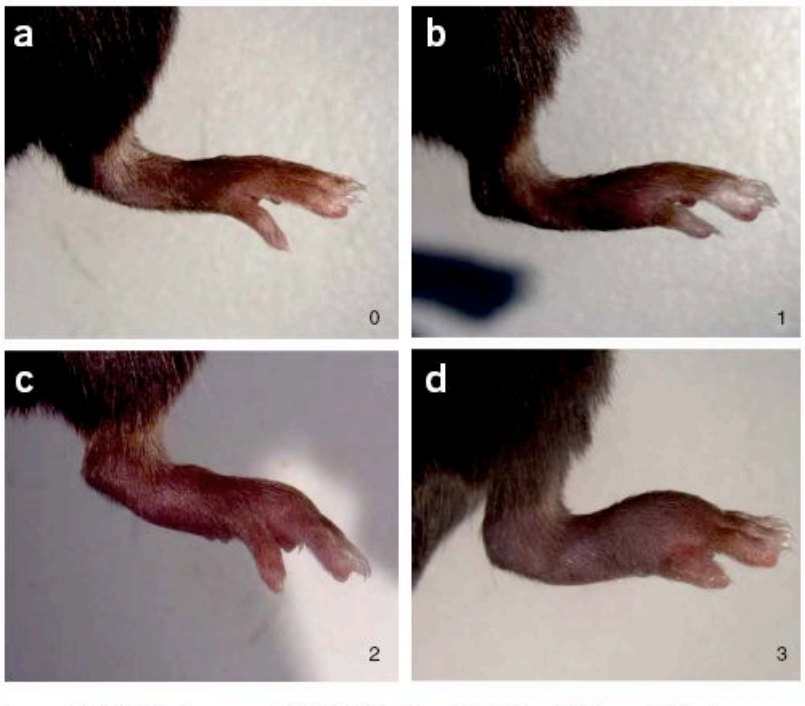

Figure 8 Clinical scores of C57BL/6 mice with CIA are assessed empirically. [41] Healthy paws (a) show no abnormal swelling, redness, contact sensitivity and motor activity alterations. The onset of arthritis with swelling of whether digits, paw or ankle is scored with 1 (a). Redness of paws must not be confused with the onset of arthritis. Pronounced limb swelling is score with 2 (c). Severe balloon-like whole paw swelling (ankylosis) is diagnosed with the maximum score of 3 (d).

3.3.5. Serum

To examine antibody levels and isotype distribution in the blood of arthritic mice, 200μl blood were collected in a serum collection centrifugation tube by carefully using

a scalpel to cut into one tail vein. Tubes were centrifuged at 6000rpm for 10min. Serum was transferred to a 1,5ml tube and stored at -80°C.

3.3.6. Statistical Analysis

Statistical analyses were done with Prism 4 (GraphPad Software). P-values were calculated using Student's *t* test: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

4. Results

4.1. Expression Vector Cloning

4.1.1. Antibody Design

For high quantity cell culture production of anti-CII antibodies we constructed the heavy and light chain V(D)J-region sequences (described in 3.1.1.) based on published data to clone them into eukaryotic murine IgG1 heavy chain and kappa light chain expression vectors. The final V-region sequences are shown for heavy chains in **Table 5**, for light chains in **Table 6**. They were ordered at MrGene and used for further cloning.

The full length open reading frame (ORF) to be transcribed into the endoplasmatic reticulum (ER) for secretion is listed for the aCII 1-5 heavy chain IgG1 vector in **Table 7**, for the aCII 1-5 kappa light chain vector in **Table 8**. The leader contains the nuclear export sequence that guides the transcribed mRNA to the rough ER where the polypeptide chain is directly translated into the ER. Before secretion the leader sequence is removed. The insertion of a restriction site between the V(D)J- and the C-region in both vectors substituted original bases without changing the number of bases or affecting antigen binding and effector function of the antibodies.

Antibody	Heavy chain sequence of variable region
aCII	>aCII-HC ACCGGTGTACATTCCcaggtgcaactgcagcagtcaggcgctgaactggcaaaacctggg acctcagtgaagatgtcctgcaaggcttctggctacaccttattagttactggatgaac tgggtaaaacagaggcctggacagggtctggaatggattggggctattaatcctagcaat ggttatactgagtacaatcagaagttcaaggacaaggccatattgactgcagacaaatcc tccagcacagcctacatgcaactgagcagcctgacatctgaggactctgcagtctattac tgtgcaagagaggactacggtagtacccactttgactactggggccaaggcaccactctc acagtctcctcaGCGTCGAC
M2139	>M2139-HC ACCGGTGTACATTCCcaggtccagctgcagcagtctggggctgaactggcaagacctgg acctcagtgaagatgtcctgcaaggcttctggctacgcctttattagctactggatgaac tgggtaaaacagaggcctggacagggtctggaatggattggggctattaatcctagcgat ggttatactgagtacaatcaaaagttcaaggacaaggccataatgactgcagacagatcc tccagcacagcctacatgcaactgagcagccttacatctgaggactctgcactctattac tgtgcaagatatggtggatactttgactactggggccaaggcaccactctcacagtctcc tcaGCGTCGAC
ACC1	>ACC1-HC ACCGGTGTACATTCCgaagtgaagcttgaggagtctggaggaggcttggtacaacctgga ggatccatgaaactctcttgtgctgcctctggattcacttttagtgacgcctggatggac tgggtccgccagtctccagagaaggggcttgagtgggttgctgaaattagaaacaaagtt aataatcatgcaacaaactatgctgagtctgtgaaagggaggttcaccatctcaagagat gattccagaagtgttgtctacctgcaaatgaacaacttaaaacctgaagacactggcatt tattattgtaccgggctaacgtttgactattggggccaaggcaccactctcacagtctcc tcaGCGTCGAC
GG-CIIC1	>GG-CIIC1-HC ACCGGTGTACATTCCcaggtccaactgcagcagcctggggctgacctggtgaggcctggg gtttcagtgaagctgtcctgcaaggcttctggctacaccttcaccagctactggatgaac tgggtgaagcagaggcctggacaaggccttgagtggattggcatgattcatccttccgat agtgaaactaggttaagtcagaagttcaaggacaaggccacattgactgtagacaaatcc tccagcacagcctacatgcagctcagcagcccgacatctgaggactctgcggtctattac tgtgcaagattgaaacccgggggcacctggtttgcttactggggccaagggactctggtc actgtctctgcaGCGTCGAC
E-CIIC1	>E-CIIC1-HC ACCGGTGTACATTCCcaggtccaactgcagcagcctggggctgacctggtgaggcctggg gtttcagtgaagctgtcctgcaaggcttctggctacaccttcaccagctactggatgaac tgggtgaagcagaggcctggacaaggccttgagtggattggcatgattcatccttccgat agtgaaactaggttaagtcagaagttcaaggacaaggccacattgactgtagacaaatcc tccagcacagcctacatgcagctcagcagcccgacatctgaggactctgcggtctattac tgtgcaagattgaaacccgagacctggtttgcttactggggccaagggactctggtcact gtctctgcaGCGTCGAC

Table 5 Heavy chain VDJ region sequences. The sequences are denoted in the Fasta file format where every line harbours 60 nucleotides. Small letters indicate V(D)J region sequences. The leader sequence and the connection to the constant parts are depicted in large letters and are underlaid in yellow. Restriction sites for cloning are underlined (HC: AgeI, SalI). Bright orange sequences are V- or J-segments that have been added to complete the published VDJ region sequence (white background) according to the germ line sequences to obtain full-length genes. Corrections of the published V-region sequences back to the germ line sequence or corrections according to the amino acid sequence are shown intense orange. GG-CIIC1 HC and E-CIIC1 HC differ only in 2 but fundamentally different amino acids. Published nucleotide and amino acid sequences were controversial. Both HC go together with the CIIC1 LC.

Antibody	Light chain sequence of variable region
aCII	>aCII-LC ACCGGTGTACATTCAgacatccagatgactcagtctccagcctccctatctgcatctgtg ggagaaactgtcaccatcacatgtcgagcaagtgagaatatttacagttatttagcatgg tatcagcagaaacagggaaaatctcctcagctcctggtctataatgcaaaaaccttagca gaaggtgtgccatcaaggttcagtggcagtggatcaggcacacagttttctctgaagatc aacagcctgcagcctgaagattttgggagttattactgtcaacatcattatggtactcct cggacgttcggtggagggaccaagctggaaatcaaaCGTACG
M2139	>M2139-LC ACCGGTGTACATTCAgacattgtgctcacccaatctccagcttctttggctgtgtctcta gggcagagagccaccatctcctgcagagccagtgaaagtgttgaatattttggcacgagt ttaatgcagtggtaccaacagaaaccaggacagccacccaaactcctcatctatgctgca tccaacgtagaatctggggtccctgccaggtttagtggcagtgggtctgggacagacttc agcctcaacatccatcctgtggaggaggatgatattgcaatgtatttctgtcagcaaagt agggaggttccgtacacgttcggaggggggaccaagctggaaataaaaCGTACG
ACC1	>ACC1-LC ACCGGTGTACATTCAgatattgtgctaactcagtctccagcttctttggctgtgtctcta gggcagagggccaccatctcctgcagagccagcgaaagtgttgataattatggcattagt tctatgaactggttccaacagaaagcaggacagccacccaaattcctcatctatgctgca tccaagcaaggatccggggtccctgccaggtttagtgggagtgggtctgggacagacttc agcctcatcatccatcctgtagaggaggatgacactgcagtgtatttctgtcagcaaagt aaggggttccgtacacgttcggaggggggaccaagctggaaataaaaCGTACG
CIIC1	>CIIC1-LC ACCGGTGTACATTCAgacattgtgatgacccaatctccagcttctttgactgtgtctcta gggcagagggccaccatatcctgcagagccagtaaaagtgttgatagttatggcaatagt tttatggaatggtaccaacagaaaccaggacagccacccaaactcctcatctatcgtgca tccaacctagaatctgggatccctgccaggttcagtggcagtgggtctcggacagacttc accctcaccattaatcctgtggaggctgatgatgttgcaacctattactgtcaacaaagt aatgaggatccgtacacgttcggaggggggaccaagctggaaataaaaCGTACG

Table 6 Corresponding light chain V-region sequences. Annotations are according to **Table 5**. Restriction sites for cloning are underlined (LC: AgeI, BsiWI). Flanking germ line sequences that have been added to the published sequence (white background) are shown in bright green. Corrections of the published V-region sequences are shown intense green. The CIIC1 LC goes with either the GG-CIIC1 HC and the E-CIIC1 HC.

Antibody	IgG1 heavy chain full length sequence of C57/B6 mouse strain
aCII	>aCII-IgG1 ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCcag gtgcaactgcagcagtcaggcgctgaactggcaaaacctgggacctcagtgaagatgtcc tgcaaggcttctggctacacccttattagttactggatgaactgggtaaaacagaggcct ggacagggtctggaatggattggggctattaatcctagcaatggttatactgagtacaat cagaagttcaaggacaaggccatattgactgcagacaaatcctccagcacagcctacatg caactgagcagcctgacatctgaggactctgcagtctattactgtgcaagagaggactac ggtagtacccactttgactactggggccaaggcaccactctcacagtctcctcaGCGTCG ACGACACCCCCATCTGTCTATCCACTGGCCCCTGGATCTGCTGCCCAAACTAACTCCATG GTGACCCTGGGATGCCTGGTCAAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAAC TCTGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTAC ACTCTGAGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGCCCAGCCAGACCGTCACCTGC AACGTTGCCCACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGT GGTTGTAAGCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCA AAGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGAC ATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGTGCAC ACAGCTCAGACGAAACCCCGGGAGGAGCAGATCAACAGCACTTTCCGTTCAGTCAGTGAA CTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGCAGGGTCAACAGT GCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACCAAAGGCAGACCGAAGGCT CCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGATGGCCAAGGATAAAGTCAGTCTG ACCTGCATGATAACAAACTTCTTCCCTGAAGACATTACTGTGGAGTGGCAGTGGAATGGG CAGCCAGCGGAGAACTACAAGAACACTCAGCCCATCATGGACACAGATGGCTCTTACTTC GTCTACAGCAAGCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAATACTTTCACCTGC TCTGTGTTACATGAGGGCCTGCACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCT GGTAAATGACGCGTACG

Table 7 Example of complete anti-CII IgG1-HC antibody gene. Annotations are according to Table 5**.** Start and stop codon are shown in red. Restriction sites for cloning are underlined (HC: AgeI, SalI, BsiWI). The V-region (small letters) was cloned between the AgeI (A^CCGGT) and the SalI (G^TCGAC) restriction sites into the vector backbone (large letters) containing part of the leader sequence and the IgG1-HC C-region described for the C57Bl/6 mouse strain followed by the BsiWI (CGTACG) restriction site.

Antibody	kappa light chain full length sequence of C57/B6 mouse strain
aCII	>aCII-kappa ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAgac atccagatgactcagtctccagcctccctatctgcatctgtgggagaaactgtcaccatc acatgtcgagcaagtgagaatatttacagttatttagcatggtatcagcagaaacaggga aaatctcctcagctcctggtctataatgcaaaaaccttagcagaaggtgtgccatcaagg ttcagtggcagtggatcaggcacacagttttctctgaagatcaacagcctgcagcctgaa gattttgggagttattactgtcaacatcattatggtactcctcggacgttcggtggaggg accaagctggaaatcaaaCGTACGGATGCTGCACCAACTGTATCCATCTTCCCACCATCC AGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCC AAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAAC AGTTGGACTGATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTG ACCAAGGACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCA ACTTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTGAAAGCTT

Table 8 Example of corresponding anti-CII kappa-LC antibody gene. Annotations are according to Table 5. Start and stop codon are shown in red. Restriction sites for cloning are underlined (LC: AgeI, BsiWI, HindIII). The V-region (small letters) was cloned between the AgeI (A^CCGGT) and the BsiWI (C^GTACG) restriction sites into the kappa vector backbone (large letters) containing part of the leader sequence and the kappa-LC C-region described for the C57Bl/6 mouse strain followed by the HindIII (A^AGCTT) restriction site.

4.1.2. Plasmid Preparation

V-region sequences of IgH (**Table 5**) and IgL (**Table 6**) were ordered at MrGene, then cloned and colonies were prepped and sequenced as described in **3.1.**. If sequencing revealed perfect sequence match with ordered sequences in an alignment BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the vectors were quantitatively propagated for cell culture transfection.

4.2. Antibody Expression

4.2.1. HEK293T Expression Machines

Corresponding IgH and IgL vectors were co-transfected, and cells receiving at least one of both plasmids were capable of producing fully functional tetrameric antibodies containing 2 heavy and 2 light chains. Transfection efficiency was assessed by measuring the antibody concentration in the supernatant via concentration ELISA. Antibody dilution series of known concentrations revealed antibody concentration in the supernatant samples via inserting colorant intensity into the standard curve. Hereby expression was proofed for all antibodies, which confirmed functionality of our vector constructs.

Additional co-transfection with a GFP expressing vector was used for optimizing transfection efficiency via directly analyzing plasmid containing cells under fluorescence microscopy.

After sterile filtration to remove residual cell contamination, antibodies in the supernatant were retained and eluted in a protein G sepharose column (**3.2.3.)**. Final protein yield after buffer exchange to PBS was determined on a NanoDrop® photospectrometer. The purification step yielded more than 90% of the initial amount of antibodies in the supernatant.

Interestingly the different anti-CII antibodies showed markedly different expression yield (**Table 9**) though all antibody chains had identical constant region sequences and plasmid vector backbones. Obviously HEK293T cells, which are commonly used

as protein expression machineries, prefer the expression of certain amino acid sequences to others. V-region amino acid sequences may influence protein folding and interaction with ribosomes, ER, chaperones, modification enzymes and the secretion machinery. Cells transfected with aCII-1-5 and M2139 antibodies showed very high expression yield compared to transfection with other antibodies. Comparability was achieved by transfecting antibodies in parallel.

IgG1 Antibody	Expression [mg / plate]
aCII 1-5	0,250
M2139	0,700
ACC1	0,100
CIIC1-GG	0,050
CIIC1-E	0,200

Table 9 Expression efficiency of anti-CII antibodies in HEK293T cells. The amount of antibody yield per transfected plate of 70% confluent cells is the mean value of more than 500 transfections where several batches of different antibodies were transfected in parallel.

4.2.2. Antibody Quality

To verify the quality of the purified antibodies a denaturizing reducing SDS-PAGE separated the subunits of every antibody.

This expression quality control showed that all anti-CII antibodies were expressed in their correct tetrameric conformation consisting of 2 heavy and 2 light chains. The single IgH ran at about 50kDa – depending on the V-region sequence length – the IgL at about 25kDa. We therefore assumed that all antibody subunit chains must have been assembled to form a native antibody, because Protein G sepharose purification only retains fully folded IgG heavy chain dimers. Retention of light chains in the column could have only been possible, if they were attached to the heavy chain dimer to form the antibody tetramer.

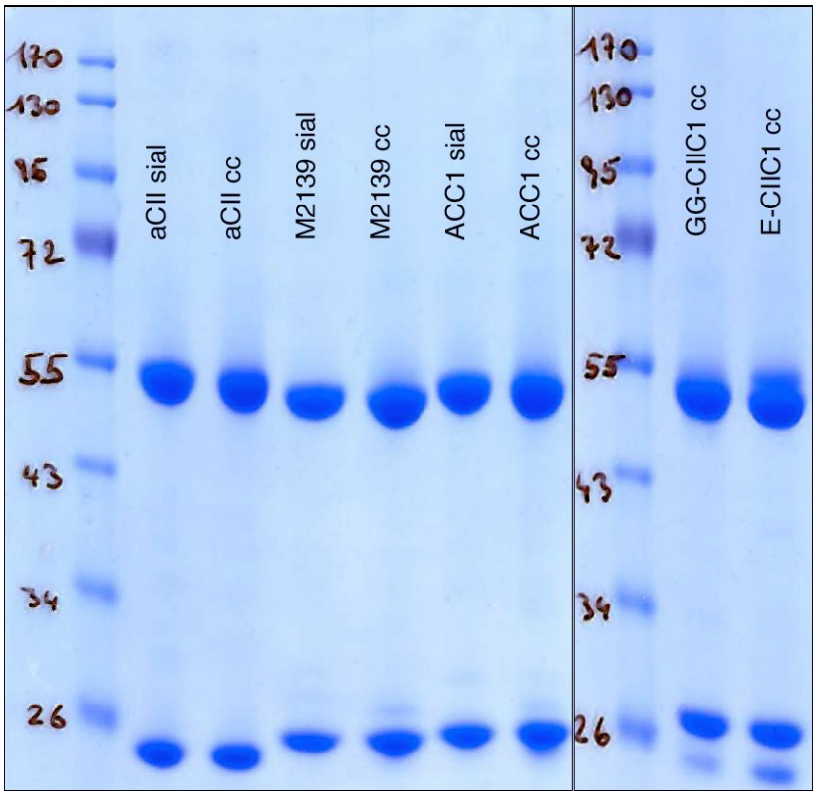

Figure 9 SDS-PAGE analysis of anti-CII antibody conformation. Denaturisation and breakage of disulfide bonds separated the 2 heavy and 2 light chains from each other. This quality control shows that every subunit of the antibodies was expressed, and that antibodies were secreted after transfection in a tetrameric confirmation. *sial* indicates antibodies after enzymatic galactosylation and sialylation. *cc* denotes cell culture purified antibodies before enzymatic glycosylation. Every gel was run with a protein standard containing peptides of known weight to conclude protein weight in the samples. Weight is indicated in Kilodalton (kDa) from 170kDa to 26kDa. One Dalton is the molecular weight of one hydrogen atom.

4.2.3. Enzymatic Glycosylation of Antibodies

To determine the Fc-glycan structure, oligosaccharides were cleaved by the bacterial enzyme EndoS, which specifically cleaves the N-linked Fc-oligosaccharide between the first and the second N-acetylglucosamine. EndoS is a defence enzyme secreted by *Streptococcus pyogenes*. Cleavage of the complete Fc-oligosaccharide abrogates IgG effector function, thus anti-bacterial antibodies lose their pathogen clearance properties. [13]

We used EndoS to cleave the Fc-glycans of the anti-CII antibodies for analyzing them in the mass spectroscopy.

Antibodies produced by HEK293T cells had short oligosaccharides lacking terminal sialic acid. About two thirds of the bisecting Fc-glycans were in the G0 glycoform terminating with N-acetylglucosamines.

However, this short IgG Fc-glycans lacking terminal sialic acid and galactose were mainly associated with proinflammatory properties. To test the antiinflammatory potential of sialylated antibodies we had to enzymatically galactosylate the terminal N-acetylglucosamines with β-1,4-galactosyltransferase and further sialylate them with β-1,6-sialyltransferase. The more efficient terminal N-acetylglucosamines were galactosylated the more binding sites for sialic acids were available. After optimizing reaction conditions for the enzymatic glycosylation assay we were able to generate antibodies with Fc-glycans of which more than 50% harboured terminal sialic acid.

Enzymatic glycosylation was first performed with the aCII-1-5 and the M2139 antibody, which yielded highest expression amount in cell culture, whereby the M2139 antibody additionally had a higher Fc-glycosylation rate than the aCII 1-5 in the first glycosylation assay though both had identical Fc-region sequences. The M2139 additionally had the highest expression rate in cell culture. We used those two antibodies for treating arthritic mice in a first therapeutic trial.

In the second enzymatic glycosylation reaction all antibodies (aCII 1-5, M2139 and ACC1) had more than 50% sialylated isotypes. All 3 antibodies were used in the second treatment.

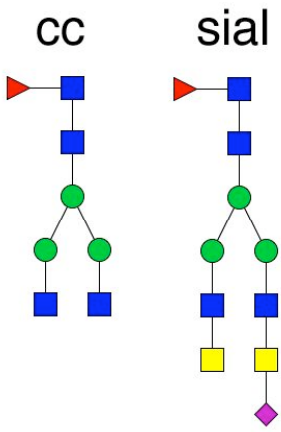

Figure 10 Fc-glycan structure of antibodies produced in cell culture (cc) and after enzymatic glycosylation (sial). Antibodies produced by HEK293A cells predominantly have short Fc-glycans with terminal N-acetylglucosamine. To test for the antiinflammatory potential of sialylated IgG antibodies, enzymatic attachment of galactose and sialic acid was required.

Antibody	total G0 %	total G1/2 %	total S1/2 %	Treatment
aCII 1-5 cc	67,85%	32,15%	0,00%	Ctrl
M2139 cc	60,94%	39,07%	0,00%	
aCII 1-5 sial	40,50%	43,97%	15,54%	1.Trial
M2139 sial	15,65%	65,89%	18,46%	
aCII 1-5 sial	5,75%	43,24%	51,00%	2.Trial
M2139 sial	5,28%	43,38%	51,33%	
ACC1 sial	6,82%	41,77%	51,41%	

Table 10 IgG1 Fc-glycosylation pattern. Anti-CII antibodies aCII-1-5 and M2139, which had a high expression yield in cell culture (cc), were enzymatically glycosylated (sial). This table shows glycosylation patterns indicating the percentage of Fc-glycan isoforms of different monoclonal antibodies. Fc-oligosaccharides contain terminal N-acetylglucosamine (G0), one or two terminal galactoses (G1/2) and one or two terminal sialic acids (S1/2). For the treatment of arthritic mice we used cell culture antibodies as a control. For the first treatment of arthritic mice (1.Trial) we used antibodies of which more than 15% of the Fc-glycans were sialylated. After optimizing glycosylation conditions we could treat arthritic mice (2.Trial) with anti-CII antibodies of which more than 50% contained sialylated Fc-glycans. Less than 7% were in the proinflammatory G0 isoform.

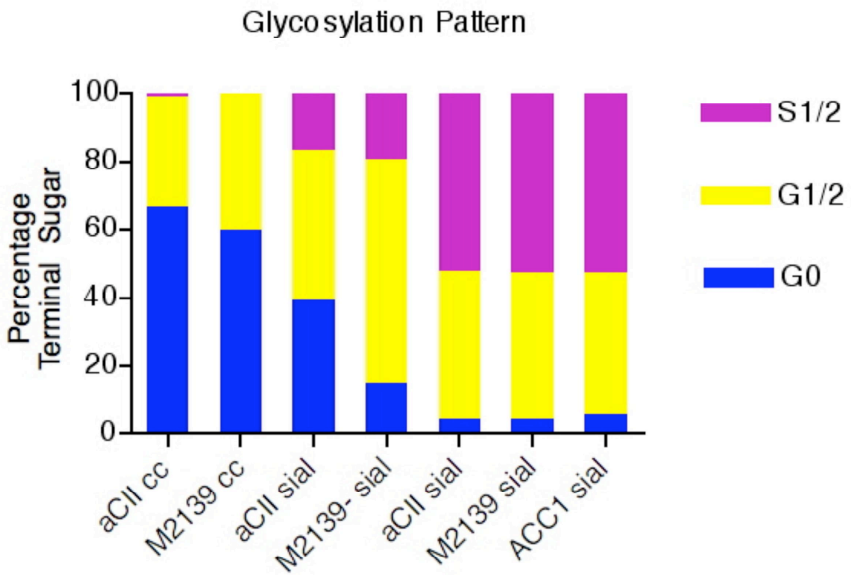

Figure 11 IgG1 Fc-glycosylation pattern. This graph depicts the relative amount of Fc-glycan isoforms of the different monoclonal anti-CII antibodies before (cc) and after enzymatic glycosylation (sial) shown in **Table 10**. After further optimizing enzymatic reaction conditions, we could treat arthritic mice (2.Trial) with antibodies, where more than 90% of Fc-glycans were terminally galactosylated (G1/2) or sialylated (S1/2).

4.2.4. Reactivity Properties of Anti-Collagen II Antibodies

Though some reactivity properties of the anti-CII antibodies have been described [35, 36], we verified collagen binding of the anti-CII IgG1 constructs to collagen type II of different species (**3.2.7.**). Trimeric CII proteins consisting of three identical α-collagen subunit chains non-covalently wrapped around each other to form a triple helix assembled into long fibrils and fibers that contribute to the viscosity of the synovial fluid in limb joints of mammals. CII is a conserved protein throughout mammalian species with a repeating core amino acid motif having a Glycin on every third position. Several immunodominant epitopes on CII have been shown to exist exclusively for B-cells or T-cells. Nevertheless CII proteins differ among species, leading to unique species specific epitopes for whether B- or T-cells. [35, 42, 43]

CII of mouse, chicken, human and bovine origin was coated on ELISA plates. All antibodies were tested for their reactivity against all CII species and characterized on an affinity plot (**Figure 12**). The better binding of one antibody to one CII was, the

lower was the antibody concentration to keep the antibody bound to CII during washing. Binding affinity was characterized by the optical density (OD). The more antibodies were bound to CII, the more HRP-coupled antibodies could bind to the anti-CII antibodies and the more colorant could be developed, which was then measured photometrically. As a negative control we used an IgG1 antibody, produced via the same expression vector cloning approach as the anti-CII antibodies, binding TNP-modified proteins – a modification motif that is not present in any CII.

The M2139 antibody showed highest expression yield in cell culture and was the best to be glycosylated in the first glycosylation assay. Now it showed highest binding affinity to CII of mouse, chicken and bovine origin compared to other anti-CII antibodies.

Interestingly, the aCII-1-5 antibody showed highest binding affinity to human CII probably due to the immunization protocol using human CII to generate this antibody, but binding to chicken and bovine CII was completely abrogated, indicating that the aCII-1-5 bound a different CII epitope than M2139. Importantly the aCII-1-5 bound mCII with high affinity too. Therefore, additionally to their properties of high expression yield, aCII and M2139 were chosen first for the treatment of arthritic mice.

The ACC1 was described to bind denatured and citrullinated CII. During inflammation of joints, necrotic cells release intracellular peptidyl arginine deaminase (PAD1), which leads to citrullination of extracellular CII in the inflammed tissue. Citrullination disables CII to form proper Collagen helices and fibrils and therefore results in the denaturation of CII and the progression of an arthritic phenotype. [36] ACC1 showed binding to all CII species. High binding of ACC1 was achieved by denaturation of mouse CII by heating it for 10 minutes at 94°C. Denaturation of native CII helices and fibrils disrupted binding properties of all other antibodies (Data not shown). To attenuate the progression of severe arthritis we included ACC1 in the treatment cocktail (aCII-1-5, M2139) for the second trial.

GG-CIIC1 and E-CIIC1 antibodies showed worse expression yield, had lower binding affinities to human and mouse CII than other anti-CII antibodies (aCII-1-5, M2139, ACC1) and showed no binding against chicken and bovine CII. E-CIIC1 all together showed similar binding properties to aCII-1-5, but at lower affinity. To reduce the

amount of antibodies with poor binding affinities, we excluded GG-CIIC1 and E-CIIC1 from the treatment cocktail.

To verify that enzymatic Fc-glycosylation of the antibodies purified from the cell culture did not influence their antigen binding affinities, we compared binding properties of the antibodies before and after enzymatic glycosylation (**Figure 13**). ELISA testing was performed analogue to **Figure 12**. Binding affinities of different Fc-glycan isoforms of aCII-1-5, M2139 and ACC1 to mouse, chicken, human and bovine CII revealed no binding propertiy differences. aCII-1-5 and M2139 produced in cell culture had short Fc-glycan isoforms (G0). They showed similar binding properties after enzymatic Fc-glycosylation. Fc-sialylation of 18 or 50% did not influence collagen binding. ACC1 before sialylation did not bind any collagen, probably due to incorrect sample handling. Anyway, ACC1 was included in the second treatment cocktail.

Results in **Figure 13** were consistent with results of **Figure 12** and proofed binding of our anti-CII antibody constructs to different CII species and thus qualified them for testing our hypothesis of treating arthritic mice with anti-CII binding antibodies harbouring anti-inflammatory properties.

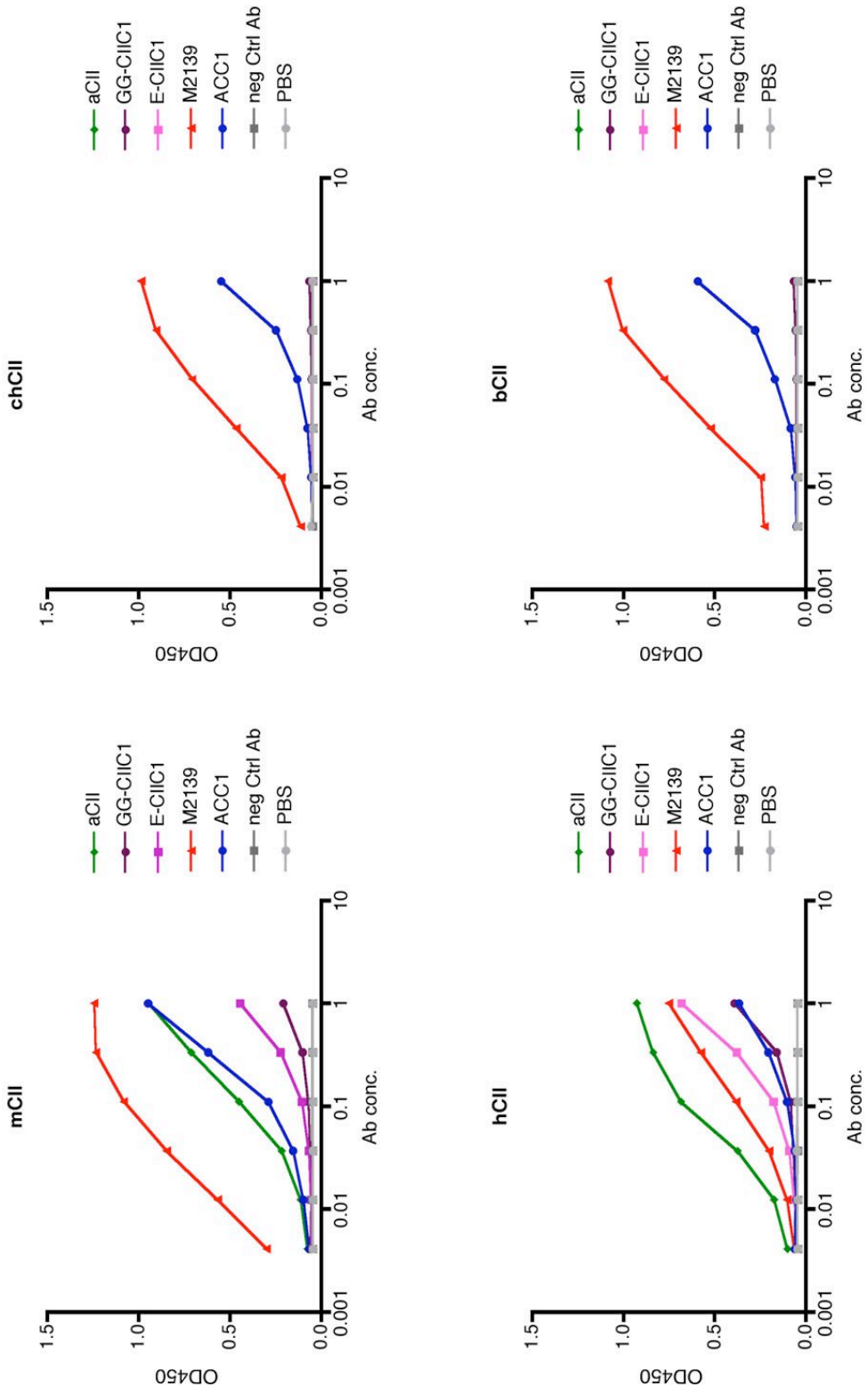

Figure 12 Reactivity Pattern of anti-CII antibodies against different CII species. Binding affinities of anti-CII IgG1 antibodies to mouse (mCII), chicken (chCII) human (hCII) and bovine (bCII) CII are shown. Optical density at 450nm (OD450) was measured at different anti-CII antibody concentrations (Ab conc.). *aCII* refers to the aCII-1-5 antibody.

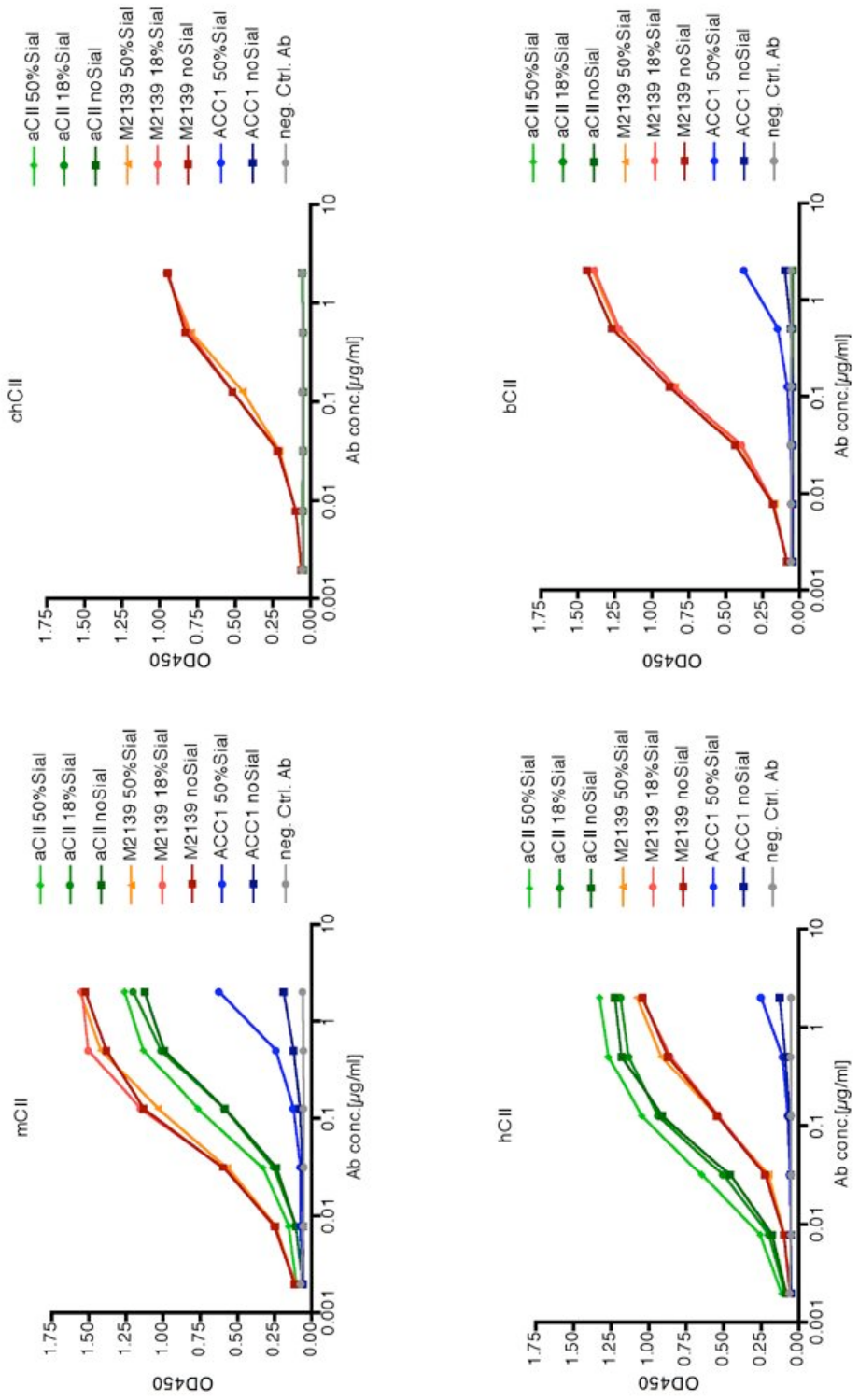

Figure 13 Reactivity Pattern of different Fc-glycan isoforms of anti-CII antibodies (aCII-1-5, M2139, ACC1). Fc-glycosylation pattern did not influence binding affinities of antibodies to CII species. IgG1 harvested from the cell culture (NoSial) showed same binding properties to CII species than after enzymatic glycosylation. Fc-sialylation (50%Sial or 18%Sial) did not change Fab binding properties. Binding affinities of anti-CII antibodies to mouse (mCII), chicken (chCII) human (hCII) and bovine (bCII) CII are indicated by optical density at 450nm (OD450) at different anti-CII antibody concentrations (Ab conc.).

4.3. Treatment of Arthritic Mice with Sialylated Antibodies

4.3.1. The Collagen Induced Arthritis Mouse Model

Collagen-induced arthritis (CIA) in mice is an important disease model of human rheumatoid arthritis (RA), which is characterized by autoreactive B- and T-cells against synovial tissue components like collagen type II (CII). [44] Autoantibodies produced by Plasma B-cells are active through Fc-receptors on immune cells like macrophages and by activation of the complement system via binding of C1q. Susceptibility to CIA and RA is associated with the expression of certain MHC haplotypes [39], whereby tolerance in non-susceptible mouse strains can be broken by increasing the amount of avirulent inactivated *Mycobacterium tuberculosis* (H37Ra) in complete Freund's adjuvants (CFA). It has been reported that H37Ra in high doses is necessary to provide enough stimulation of Toll-like receptor 2 (TLR2) [45] on dentritic cells (DC) to induce the expression of proinflammatory cytokines and to trigger immune response even in H-2^b harbouring "non-susceptible" mouse strains. [40]

However, problems inducing CIA in H-2^b mice like C57BL/6 have been described, and several protocols have been published in recent years dealing with this difficulties. Immunization time-points, booster injections, the use of CFA with different amounts of H37Ra, the use of heterologues CII species and even the contribution of the mouse facility have been topic of discussions. [39, 40, 41]

After studying literature and with great advice from Andrei Kruglov from the Sergei Nedospasov group at the DRFZ, we decided to immunize C57BL/6 mice with chCII in CFA containing 5mg/ml inactivated H37Ra. Heterologues chCII is necessary to overcome tolerance against self-CII as it would be the case with the more homologues bCII. A second booster immunization at day 21 again with chCII in CFA (5mg/ml H37Ra) was necessary for the development of the arthritic phenotype.

However, about 20% of the mice developed ulcerations at sites of injection and had to be sacrificed. Our CIA protocol led to disease onset in C57BL/6 mice within one week after booster immunization. Peak incidence and severity were achieved around day 42 after the first immunization. About 50% of the C57BL/6 mice receiving the CIA protocol developed arthritis. The mean clinical value, which is a mean value measuring the severity of all arthritic limbs in a group (**3.3.4.**, **Figure 8**), reached a peak of between 3 and 4. Read out of the experiments was set at day 42 after the first immunization, because limbs with sustained severe ankylosis cripple. During that process swelling goes down and mean clinical score read out is no representative value for severity any more. After setting the protocol for C57BL/6 mice, a CIA protocol for FcγRIIB$^{-/-}$ mice was established. FcγRIIB$^{-/-}$ mice have a C57BL/6 background only lacking the inhibitory FcγRIIB$^{-/-}$ receptor on immune cells, which makes them more susceptible to CIA though carrying the H-2^{b} haplotype. [30]

It has been suggested that sialylated antibodies have antiinflammatory potential due to their altered affinity to Fcγ-receptors. Affinity of IgG antibodies to their Fcγ-receptors triggering proinflammatory cascades is lowered according to Fc-sialylation, thus leading to less inflammatory effector potential. [20] Nourished by the identification of a lectin receptor called SIGN-R1 on macrophages, that binds sialylated IgG antibodies and contributes to their antiinflammatory effects, [22] and the FcγRIIB-independent inhibition of antigen presentation and T-cell activation, [28] we suggested a novel hypothesis of an FcγRIIB-independent mechanism as the base of the anti-inflammatory activity of sialylated IgG antibodies.

We used FcγRIIB$^{-/-}$ mice for treating arthritic mice with highly sialylated anti-CII IgG antibodies to additionally look for an FcγRIIB-independent IgG effector mechanism.

We applied the same immunization protocol for FcγRIIB$^{-/-}$ mice as for the C57BL/6 except of boosting them on day 14 with incomplete Freund's adjuvants (IFA) not containing any inflammation stimulating H37Ra *Mycobacteria tuberculosis*. Up to 90% of FcγRIIB$^{-/-}$ mice developed arthritis on at least on limb with a peak mean clinical score of up to 6 (healthy and sick mice in one group together). Immunizing the mice first with chCII in standard CFA (1mg/ml H37Ra) even FcγRIIB$^{-/-}$ mice showed the robust H-2^{b} phenotype with only 30% of mice developing arthritis. No FcγRIIB$^{-/-}$ mice showed any ulcerations or wounds at the site of injection at all.

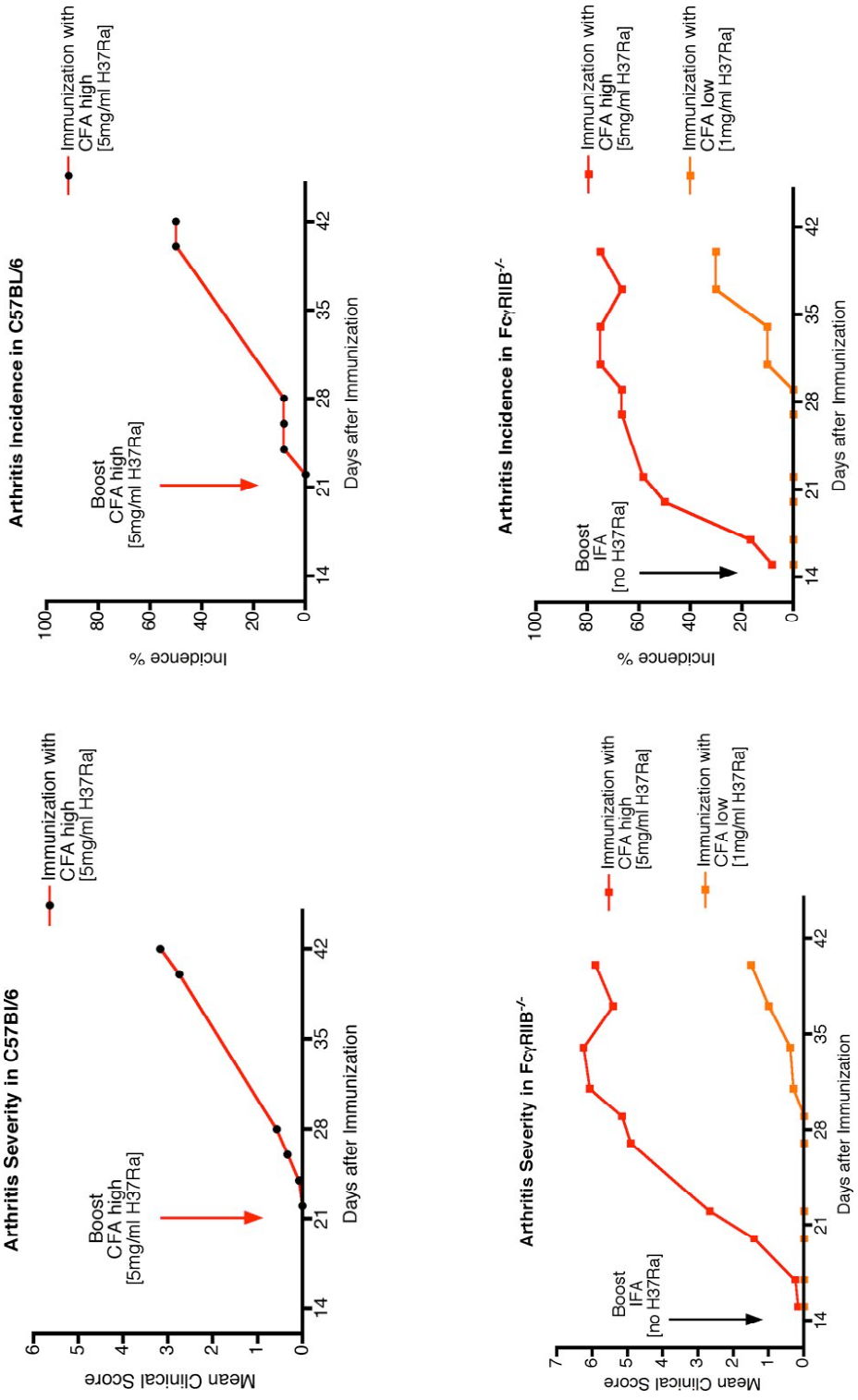

Figure 14 Arthritis severity and arthritis incidence in C57BL/6 and FcγRIIB$^{-/-}$ mice. Establishment of CIA in C57BL/6 and FcγRIIB$^{-/-}$ mice was set using different immunization protocols. The mean clinical score is a value of arthritis severity of all healthy and sick mice of one group, including all 4 limbs of every mouse (**Figure 8**). The arthritis incidence is a measure of the relative amount of arthritic mice in one group. A mouse is scored arthritic if it has at least one limb scored not healthy (>0).

4.3.2. Treatment Scheme

The induction of CIA leads to an immune response against chCII. Cross-reacting B- and T-cells trigger inflammation in host joint collagen. A self-propagating process in the inflamed joints starts and an arthritic phenotype is established. Because of the host immune reaction against self-CII, we antigen-specifically target mCII with sialylated anti-mCII antibodies to test for their anti-inflammatory activity. We used two protocols in this experiment to test for the influence of injection time-points, the amount of antibody and sialylation degree necessary to show effects. Sialylated antibodies were injected before immunization and at different time points before the boost to inhibit the development of an immune reaction against self-CII. To observe effects of intraperitoneal (i.p.) and intravenous (i.v.) administration, antibodies were injected i.p. in the first and i.v. in the second trial.

In the 1.Trial we injected twice 100μg of an antibody cocktail containing 50μg aCII 1-5 and 50μg M2139 – 15-18% of which had terminal sialic acid – in FcγRIIB$^{-/-}$ mice (n=10). An IFA boost was given at day 21. In the 2.Trial of this experiment FcγRIIB$^{-/-}$ mice (n=10) were injected twice 450μg of an antibody cocktail containing 150μg aCII 1-5, 150μg M2139 and 150μg ACC1. All antibodies in this cocktail had more than 50% terminal sialic acid.

One control group in each trial received corresponding amounts of the same but unsialylated antibody cocktail. Control antibodies were purified from cell culture supernatant and did not receive the enzymatic Fc-glycosylation protocol.

The second control group in each trial received a corresponding volume of PBS without antibodies.

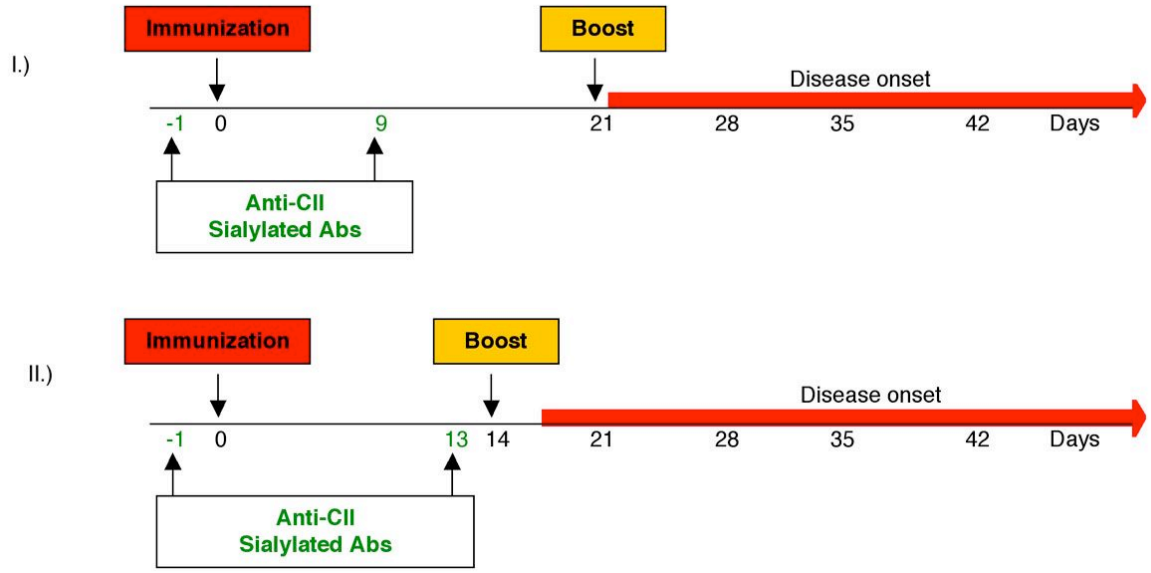

Figure 15 Treatment scheme. In the 1.Trial (I.) FcγRIIB$^{-/-}$ mice (n=10) received a total of 200μg of up to 18% sialylated antibodies (aCII 1-5, M2139) with two injections intraperitoneally. One day before and nine days after the first immunization 100μg of antibody cocktail were injected. For the 2.Trial (II.) FcγRIIB$^{-/-}$ mice (n=10) received a total of 900μg (150μg of aCII, M2139 and ACC1 each) via two injections of 450μg intravenously one day before immunization and one day before booster injection.

4.3.3. Sialylated Antibodies Attenuate Disease Progression

FcγRIIB$^{-/-}$ mice were treated with sialylated anti-CII IgG1's according to the treatment scheme in **Figure 15**. In the 1.Trial mice received less amount of antibody, which were also less sialylated; in the 2.Trial mice received a higher dose of highly sialylated antibodies. In both trials one of the control groups received corresponding amounts of non-sialylated antibodies. Second control group received PBS. Mice were observed thrice a week blinded for the development of arthritis.

Interestingly disease onset was not altered by the injection of the antibodies and started within one week after booster injection with IFA in all groups independent of the amount of administered antibodies. The second booster injection triggered disease onset in all groups, but mice receiving non-sialylated control antibodies developed severe arthritis immediately within one week. Compared to the PBS group the non-sialylated antibodies had a severity promoting effect in the early phase of

disease. As suggested by *Parekh et al* [16, 34] in the 1980's and reviewed in 2009 [12] antibodies in rheumatoid arthritis were associated with short Fc-oligosaccharides predominantly ending with terminal N-acetylglucosamine. However, in the 2.Trial high amounts of control antibodies did not promote severity in the late phase of disease.
Around day 28 PBS control mice started developing severe arthritis as seen in the establishment protocol.
In both trials mice given sialylated IgG antibodies behaved similar to PBS control mice during the first week of disease onset, but were prevented from developing severe arthritis until evaluation day 42. Even those animals receiving only 200μg of less sialylated (<18%) antibodies were prevented from developing severe arthritis. These results suggest that antibodies of which about 15% are terminally sialylated are sufficient even at lower dose to protect joints from severe arthritic inflammations. The results of both trials leave the question open if time-points of injection and boosting are more important than the amount of antibodies to be injected.

Incidence rates of mice receiving sialylated anti-CII antibodies were between 60% (1.Trial) and 70% (2.Trial) at day 42, which were 20-40% less than in both control groups. Non-sialylated IgG's with shorter Fc-glycans increased incidence rates at the beginning of the disease to be 20-40% higher around day 28, but incidence curves adjusted to those from the PBS group in the later phase of disease.

We observed that sialylated antibodies inhibited the development of severe arthritis and reduced incidence rates. However, sialylated antibodies did not prevent from disease onset. Even highly sialylated antibodies at high doses do not alter severity or incidence curves compared to lower doses of less sialylated IgG's. A sufficient amount of sialylated antibodies seems to be necessary to switch off part of the immune response.

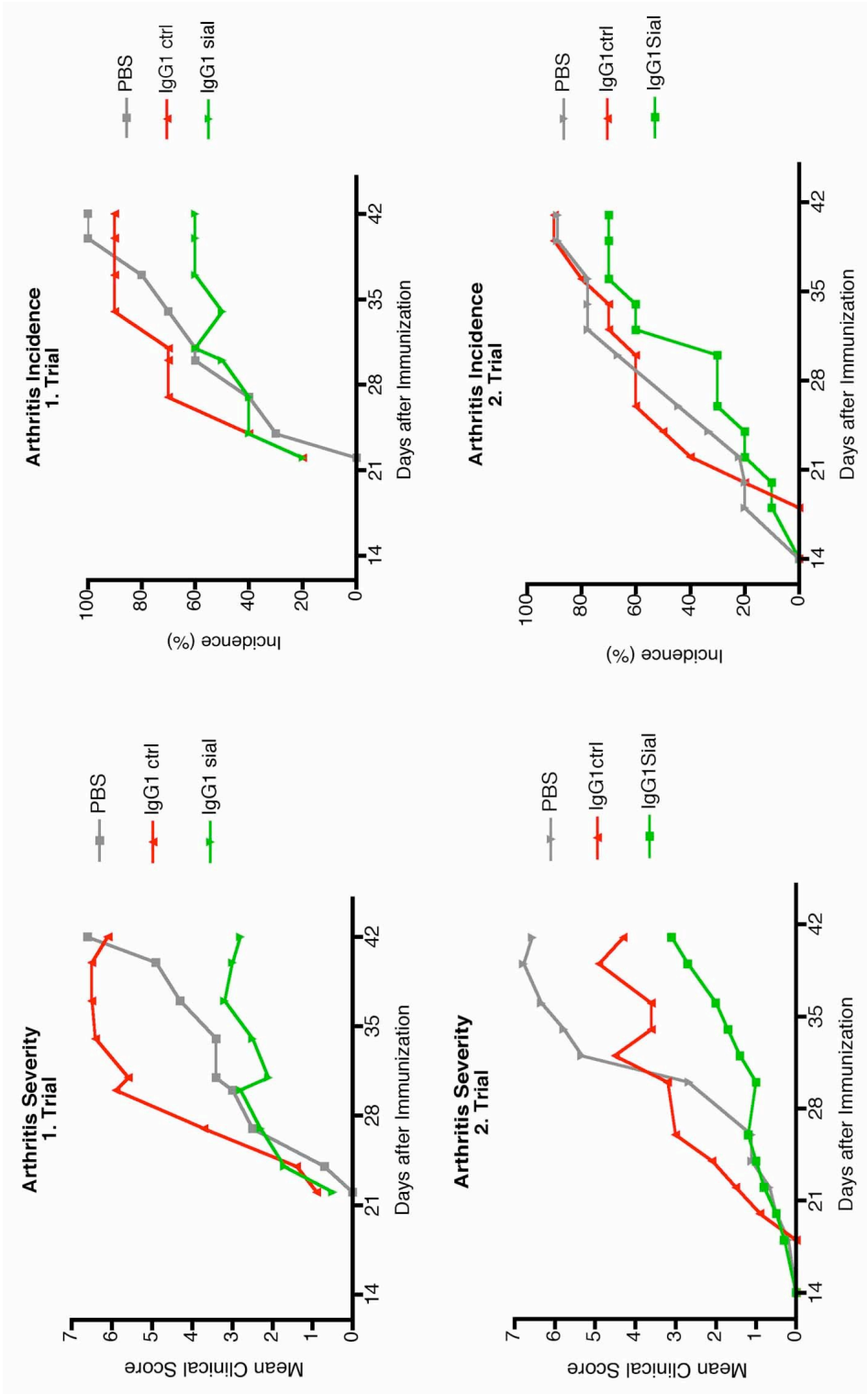

Figure 16 Severity and incidence of arthritis after treatment with sialylated antibodies. In both trials one group of mice (n=10) received a sialylated IgG1 antibody cocktail (IgG1 sial), one group (n=10) receive the corresponding non-sialylated IgG1 cocktail (IgG1 ctrl) and the last group (n=10) was the PBS control group (PBS). In the 1.Trial 200μg IgG1 sial (<18% sialylated) were administered. In the 2.Trial 900μg IgG1 sial (>50% sialylated) were given.

4.3.4. Sialylation Pattern of Serum IgG

As observed during establishment of the CIA protocol, not all of the mice developed arthritis. Depending on the immunization protocol a fraction stayed healthy.

To examine differences in the glycosylation pattern of anti-CII antibodies in mice that stayed healthy despite applying the CIA protocol and in mice that became sick after CIA immunization, we pooled equal volumes of sera from healthy mice and from sick mice out of untreated control groups, respectively. None of the mice had received any antibodies. 100μl serum of 5 healthy mice were pooled and incubated with chCII immobilized in an agarose bead column and fractions containing antibodies that had been retained in the column were pooled and used for MALDI-TOF analysis of their Fc-glycan pattern together with the whole serum IgG containing flow through that did not bind to the chCII column. To search for difference in the Fc-glycan pattern between healthy and arthritic mice, the same procedure was applied with serum IgGs of 5 severely arthritic mice (clinical score per mouse ≥10).

Results of Fc-glycan analysis of serum anti-CII and flow through IgG-glycosylation patterns claimed reduced Fc-sialylation of anti-CII IgGs as well as of flow through serum IgGs of severely arthritic mice compared to IgGs of their healthy littermates.

Anti-chCII IgG's of arthritic mice that might influence arthritic phenotype via cross reacting with self-mCII were retained in the chCII column. Alterations in the Fc-glycosylation pattern could be observed (**Figure 17**). Arthritic mice displayed more anti-chCII IgG Fc-glycans appearing in the G0 isoform (data not shown) compared with Fc-glycans of healthy mice. These results are consistent with previous findings [16, 20, 33] that desialylated IgGs promote arthritic inflammations, whereas sialylated IgGs prevent from the development of arthritis. Additionally, the results of the two trials (**4.3.3.)**, where arthritic mice were treated with sialylated and desialylated IgGs, have also led to the conclusion that sialylated IgGs can prevent from severe arthritis while desialylated IgGs promote inflammation.

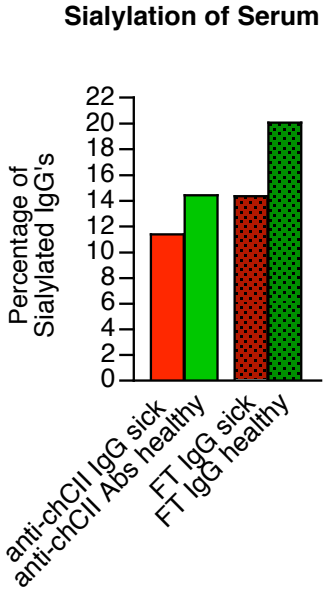

Figure 17 Fc-sialylation of serum IgG's of healthy and severely arthritic mice. Pooled serum IgG's that were retained in a chCII column and corresponding flow through (FT) IgG's of healthy mice and severely arthritic mice (clinical score per mouse ≥10) were analysed according to their Fc-sialylation pattern. Anti-CII IgG's as well as other serum IgG's of arthritic mice showed reduced Fc-sialylation.

4.3.5. Serum Levels of Ig subclasses in arthritic and non-arthritic mice

Another subject of our interest was whether Ig subclass (IgA, IgM and IgG) and IgG isotype (IgG1, IgG2a, IgG2b and IgG3) serum levels markedly differ between chicken CIA mice that had received sialylated or desialylated anti-CII antibodies or PBS. Additionally we wanted to examine, if the development of an arthritic phenotype correlates with the expression of certain Ig subclasses and isotypes.

To analyse differences in serum Ig levels of mice from different groups of the 1.Trial and the 2.Trial, serum-ELISA (**3.2.7.**) was the method of choice. Antibodies in the sera of mice were tested on mCII and chCII. Bound antibodies were detected by HRP-coupled secondary antibodies directed against different mouse Ig subclasses and IgG isotypes. Photometric signals display the amount of anti-mCII and anti-chCII antibodies of different subclasses and isotypes in the serum.

We supposed mCII to be the phenotype-causing antigen. Interestingly immune responses against mCII did not necessarily correlate with the immune response

against chCII. All ELISA data depicted in Figures 18-20 are taken from the 2.Treatment-Trial (**4.3.3.**) and are representative for all experiments. Evaluation of the serum Ig content was done at day 42 (d42) after the 1.immunization with chCII. At this time-point no elevated IgG1-level was detectable that was due to the injection of sialylated or de-sialylated anti-CII IgG1 on d-1 and d13.

First we examined the influence of sialylated anti-CII IgG1 antibodies that had been injected between d-1 and d13 on the expression of intrinsic anti-mCII and anti-chCII antibodies (IgG, IgG1, IgG2b, IgG2c, IgG3, IgM and IgA) at d42. Mice receiving sialylated anti-CII IgG1 had lower total serum IgG against mCII and chCII than the PBS control group and the group treated with de-sialylated IgG1 (anti-CII-cc) (**Figure 18**). This result is consistent with the idea of a general immunosuppressive effect of sialylated antibodies. How sialylated anti-CII IgG1 antibodies suppress the general production of new IgGs remains to be elucidated. At d42 IgM levels of mice receiving sialylated antibodies were elevated, though these date were not reproducible. However, no significant differences in the expression of different intrinsic Ig subclasses could be observed.

Additionally we investigated serum Ig levels of healthy and arthritic mice. To examine, if there was a general humoral mechanism controlling the development of arthritis, we analyzed healthy mice of all 3 groups, as well as arthritic mice of all 3 groups. Arthritic mice had significantly higher levels of IgG2b and IgG2c (=IgG2a of C57BL/6) in blood serum compared to healthy mice at d42 supporting findings that these IgG subclasses have pathogenic properties in autoimmune disease. [10] Even though, mice receiving the CIA immunization protocol without displaying an arthritic phenotype had increased serum anti-mCII and anti-chCII antibody titers of different subclasses at the end of the experiment (day 42) at comparable levels to arthritic littermates, suggesting another mechanism critically regulating the switch between inflammation and tolerance (**Figure 19**). Sustained inflammation might therefore be due to high IgG2b and 2c levels.

Indications that glycosylation pattern of IgG antibodies have a major impact on the development of arthritis had come from experiment **4.3.5.**, where arthritic mice had anti-chCII antibodies with less terminal sialic acid.

Take together, the results of Ig subclass serum ELISA supported the hypothesis of a major mechanism in autoimmune arthritis concerning the Fc-sialylation of IgG antibodies that contributed to an arthritic phenotype.

One prognostically relevant observation has been made by looking at IgM levels at d12 (**Figure 20**) before the onset of arthritis between d15 and d25. Mice receiving sialylated IgG1 antibodies produced significantly higher amounts of anti-mCII IgM before the onset of the disease compared to desialylated anti-CII IgG1 and PBS control groups, but no differences could be observed concerning anti-chCII IgM. High anti-mCII IgM titers in blood serum before the onset of the disease seem to prevent from the development of arthritis. These results indicate a tightly regulated immune response against self-antigens. The initial phase of the immune response possibly predicts later clinical outcome. If sialylated anti-CII IgG1 antibodies directly trigger the early expression of IgM remains to be elucidated.

The contribution of specific immunogenic CII epitopes as well as the role of regulatory (T_{reg}) and proinflammatory (Th17) T-cells must as well be addressed for further understanding the mechanistical network that leads to the development of autoimmune CIA. Insights from disease mouse models can be an important guide for the translation of therapeutic approaches to human autoimmune diseases like rheumatoid arthritis.

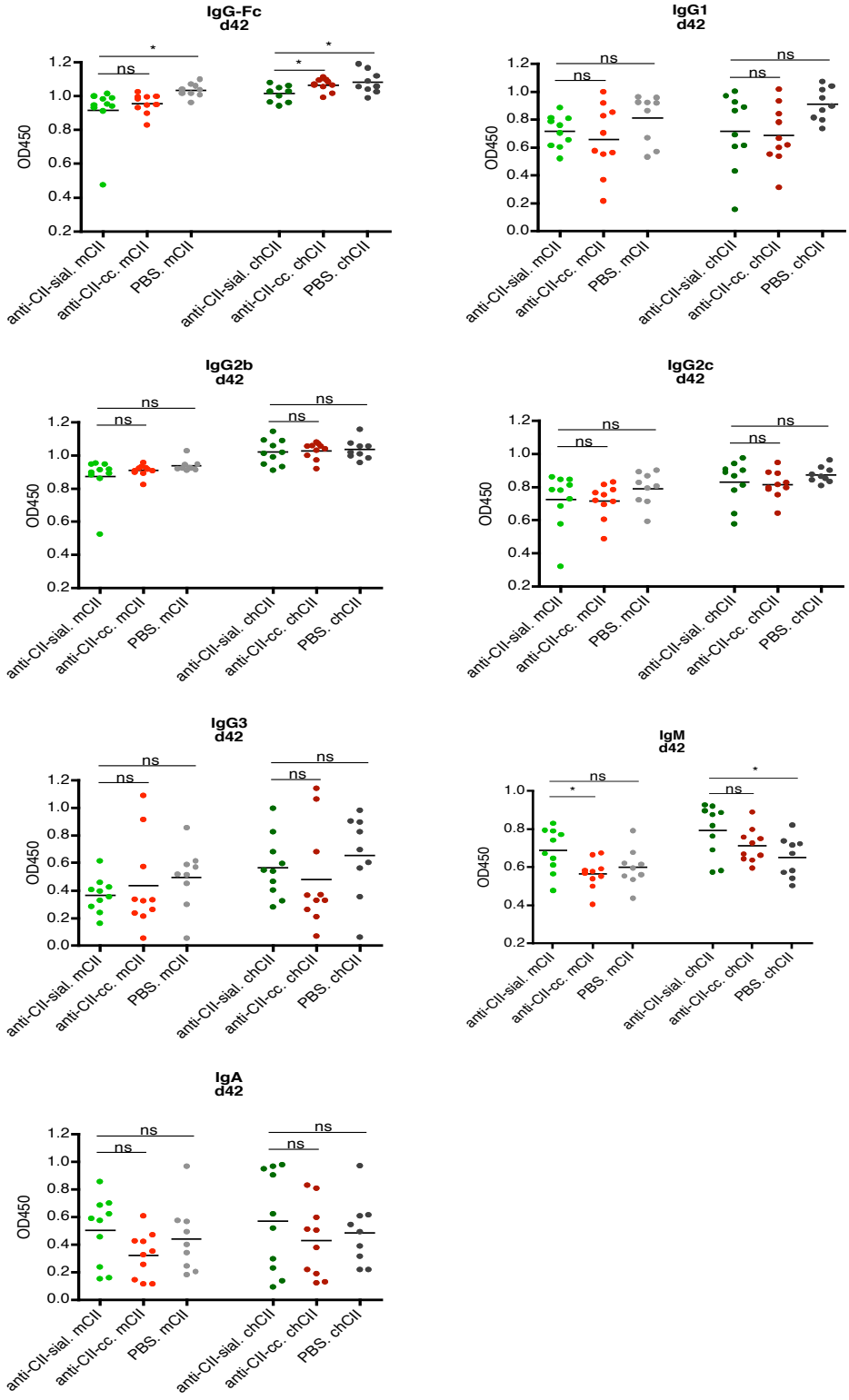

Figure 18 Serum Ig levels at day 42 of CIA FcγRIIB-/- mice treated with sialylated (anti-CII-sial) or desialylated (anti-CII-cc) IgG1 antibodies. The control group received PBS. Intrinsic serum antibodies of every mouse were tested for reactivity against mouse CII (mCII) and chicken CII (chCII). Serum Ig subclasses were detected with secondary HRP-coupled antibodies specific for different subclasses. No significant differences in Ig subclass and IgG isotype distribution could be detected that were representative for other experiments, except that general IgG levels (all isotypes) were reduced in mice that received sialylated anti-CII IgG1 compared to those mice that did not receive antibodies.

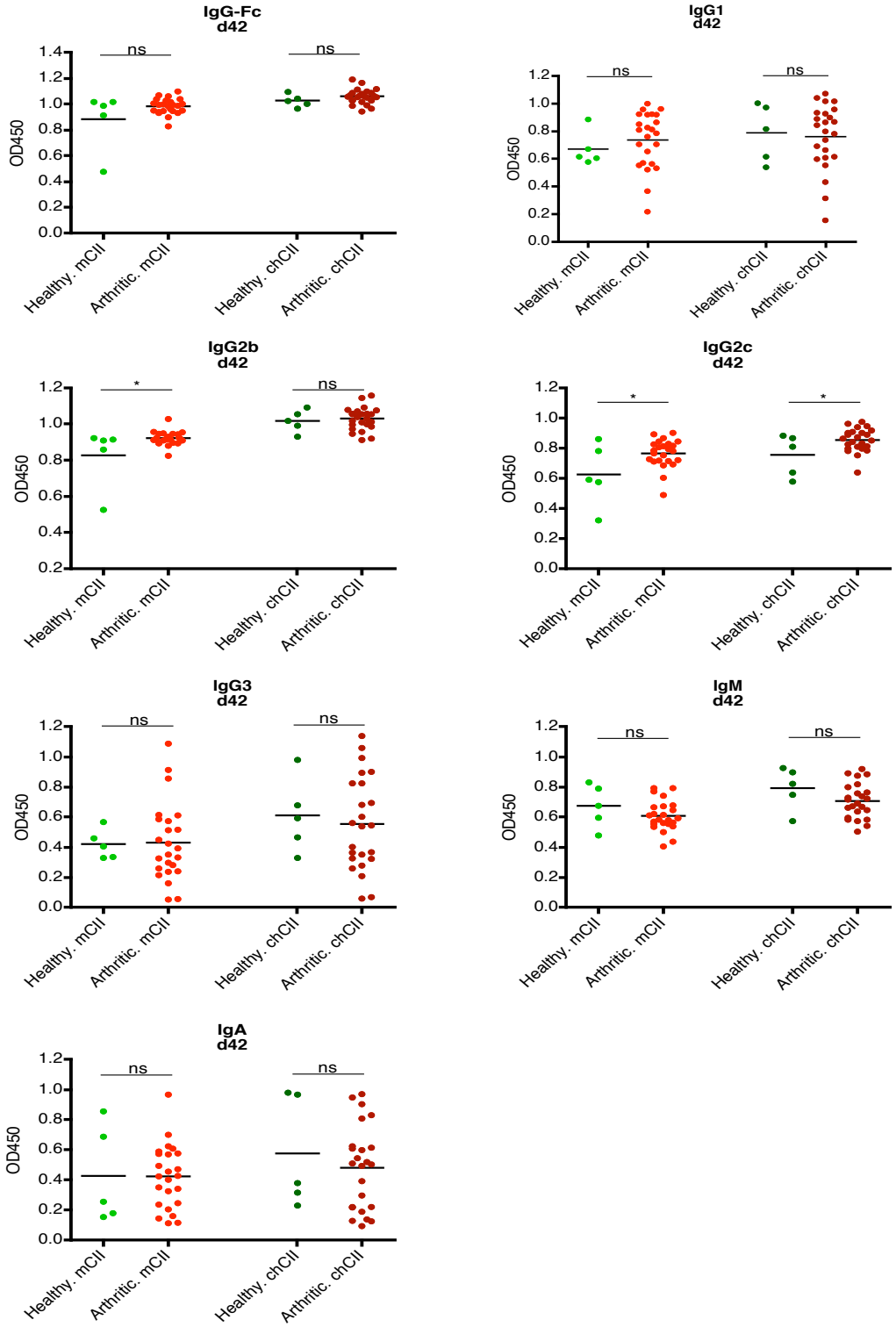

Figure 19 Serum Ig levels of healthy and arthritic mice. Serum of all healthy and arthritic FcγRIIB-/- mice from all groups (treated with anti-CII-sial, anti-CII-cc or PBS) of the 2.Trial (representative for all experiments) were taken together and tested for reactivity against mCII and chCII. Primary antibodies in the serum were detected by secondary HRP-coupled antibodies against different Ig subclasses. Reactivity to mCII was considered as phenotype relevant. Proinflammatory IgG2b and IgG2c remained upregulated in arthritic mice. No other significant differences could be observed at day 42 (d42) after immunization.

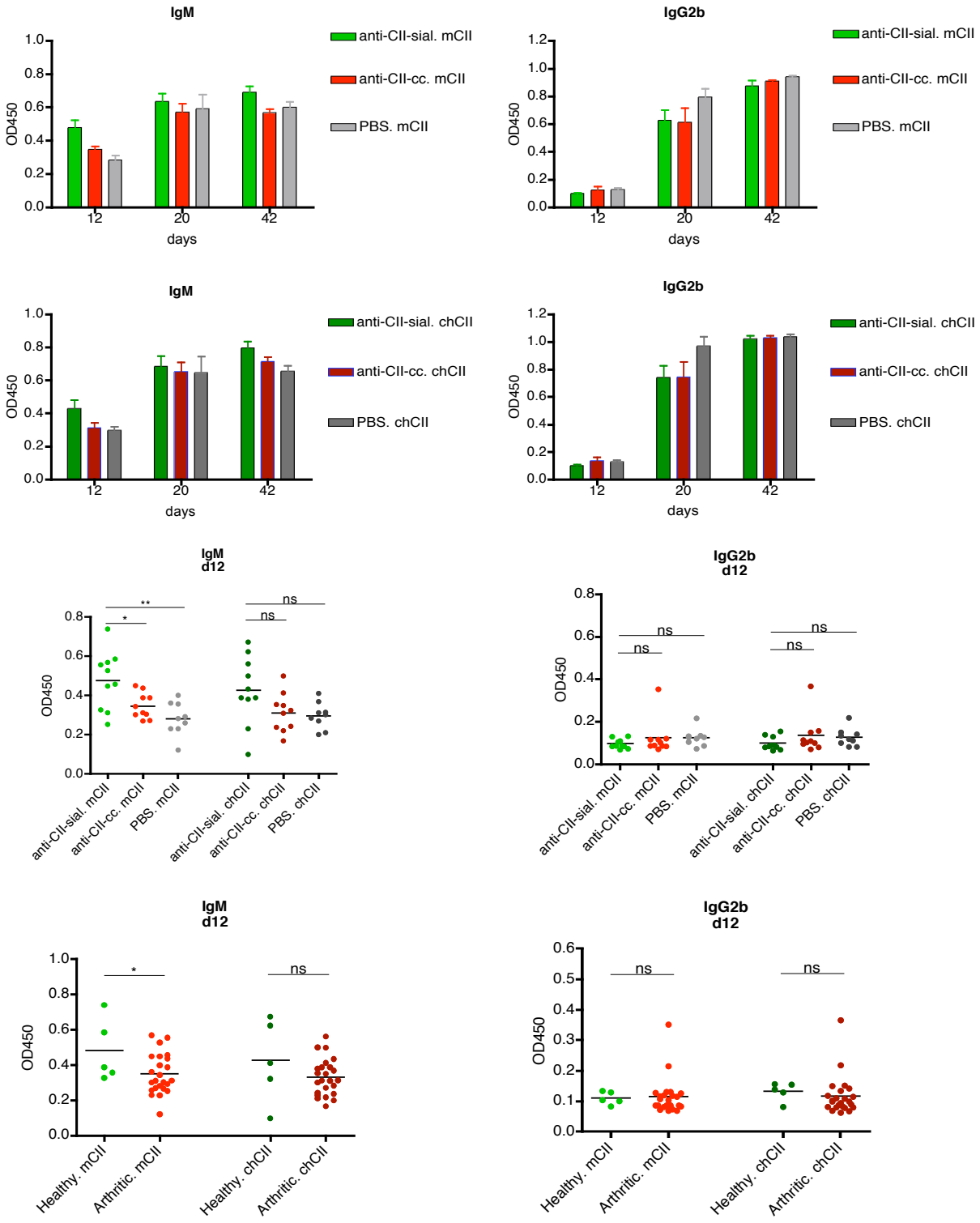

Figure 20 IgM and IgG2b serum levels over time and in detail at day 12 after immunization (d12). IgM is significantly upregulated at d12 in mice that have received sialylated antibodies (anti-CII-sial) compared to mice that have received desialylated antibodies (anti-CII-cc) or control mice (PBS). Additionally IgM is upregulated at d12 in mice that stay healthy until the end of the experiment (d42). To compare, no significant differences in proinflammatory IgG2b expression at day were found in any of the groups over different time points.

5. Discussion

This diploma project aimed to investigate the effect of sialylated IgG antibodies in a preclinical autoimmune disease mouse model. Sialic acid is a sugar, which can be attached terminally to the IgG Fc-glycan. Sialylated IgGs have been described to have novel effector functions. [7, 19, 20] Upon sialylation of the Fc-glycan, IgG antibodies switch from an activating to an inhibitory effector molecule. This effect of IVIG (serum IgG pool) was shown to be systemically translated by splenic marginal zone macrophages in mice. Due to several findings (unpublished) in our lab, there was strong indication that one could transfer this antiinflammatory property of sialylated IgG Fc's by targeting the autoimmune disease causing self-antigen with sialylated IgGs that have antigen-specific Fab regions.

Collagen-induced arthritis (CIA) is an autoimmune disease mouse model where collagen type II (CII) is the defined arthritic phenotype causing self-antigen. IgG antibodies produced by plasma cells act in concert with T-cells to promote arthritis.

5.1. Newly Designed Antibodies Bind To Antigen

To use the anti-inflammatory potential of IgGs for the treatment of CIA we designed highly sialylated anti-CII IgG1 antibodies to attenuate the host immune response against its self-CII in the synovial fluid. The V-region sequences of the antibodies were published. [34, 35, 36] A cocktail of these antibodies was known to efficiently induce arthritis at concentrations of about 9mg/mouse. [34, 43] Most of the antibodies were originally isolated from inflammatory IgG2a and IgG2b isotype expressing hybridoma

cells. [10, 35, 36] To be able to produce these antibodies separately and on different IgG subclasses like the less activating mouse IgG1 subclass [9] we cloned the antibodies.
No data about the glycosylation status of these antibodies when trying out the arthritis induction experiments were found, suggesting that IgGs were in a less sialylated form to efficiently induce arthritis.
Investigations on the degree of sialylation of our antibodies revealed that IgG1's produced in cell culture did not contain any sialylated Fc-glycans. Fc-glycan patterns of these cell culture antibodies were associated with proinflammatory effector properties [12, 16, 18, 33] due to more than 2/3 of the Fc-glycans being in the G0 glycoform.
IgG antibodies of healthy individuals show a sialylation degree of about 15%. Healthy mice examined in our study had about 20% sialylated IgGs. There exist no data if IgG subclasses are differently sialylated – neither in humans nor in mice. Therefore we suggested a sialylation degree of more than 15% in an antigen-specific IgG1 sample to be sufficient to show effects at the site of inflammation. One experiment was carried out with 18% sialylated IgG1, the 2.Trial with more than 50% sialylated IgG1.

5.2. Sialylated IgG's Prevent From Severe Arthritis

5.2.1. Antigen-Specific Prevention From Severe Autoimmune Disease

The only antiinflammatory approved products based on IgGs are IVIG (intravenous immunoglobulin G) biologics. [7, 21, 23] It has been shown that the antiinflammatory activity of IVIG is based on a small fraction of sialylated IgGs. [19, 20, 21] However, IVIG has a systemic shut-down effect on the immune system, including risk to increased susceptibility to pathogens, transfer of inadvertent immune responses and side effects including headache and fatigue, due to very high dose administration of off-target IgGs. The transfer of pooled donor samples can also lead to variations in the quality of different batches. [19, 21, 23]
We were able to show that mice receiving the CIA protocol could be prevented from developing severe autoimmune arthritis, which was evoked by immune response against self-CII, by the administration of low dose recombinant anti-CII specific sialylated IgG1 antibodies.

Consisting with the results of recent publications [11, 12, 33] we found a correlation of the effector function of monoclonal antigen-specific antibodies with their differently glycosylated Fc portions. As suggested in the analysis of IgG Fc-glycans of patients with rheumatoid arthritis [12, 18, 33] and in our observations of CII-specific IgG Fc-glycans of arthritic and non-arthritic mice, vice versa the recombinant IgGs induce pro- and antiinflammatory activities upon application. Sialylated antibodies administered before and after induction of CIA inhibited the development of arthritis compared to the control group by exhibiting less than half of the clinical score. The clinical outcome was markedly beneficial, even though mice were not completely prevented from the induction of arthritis, suggesting additional molecular and cellular contributions to the origin of disease.
The disease promoting effect of the desialylated anti-CII IgG1 hints to the binding of the antibodies to their joint targets. In vivo binding of the antibodies to CII in the synovium has been shown before. [6]

IVIG is administered at 2mg/g body weight, which 40,000μg per mouse per injection. We were able to achieve beneficial effects in arthritis therapy with up to 400-fold less amount of IgGs compared to IVIG by using sialylated antigen-specific IgG1 antibodies, even suggesting a lower dose to achieve efficient effects, because elevated IgG1 levels in blood serum can be detected up to several weeks after injection. This suggests CII-specific antibodies to be present at saturated levels in the joint structures.
Severe side effects of this therapy could not be observed by the experimenter. All mice in the different groups behaved according to their status of disease.

5.2.2. Outlook

It remains to be elucidated if administration of the sialylated autoantigen-specific IgGs after the establishment of the disease can attenuate arthritic symptoms. This question would be of particular medical relevance and would allow a more challenging comparison between approved medical therapeutics like IVIG and potential new recombinant biologics.

Another control necessary for preclinical studies would be the administration of an sialylated antigen-specific IgG1 antibody that binds no target in the mouse, to exclude off-target contributions, as well as anti-CII-specific antibodies that are completely abrogated in their Fcγ-receptor signalling due to complete removal of their Fc-glycan by EndoS. [14] This could also point out differences in the proposed antiinflammatory signalling triggered by sialylated antibodies compared to a total abrogation of signalling due to the loss of the Fc-glycan. Concerning this, trials would proof that not only sialylated activation-failing IgGs in this model just simply block arthritogenic epitopes on CII to avoid binding of cytotoxic antibodies and activating T-cells and therefore inhibiting the establishment of severe arthritic phenotypes; but that inhibitory signalling by sialylated antibodies is actively triggered. At least Fc-sialylation decreases the binding of IgG1 to the activating Fcγ-receptor III only by about 10-fold [20], but does not abrogate their interaction completely. Other interactions could though be blocked, because no activating IgG2a or 2b subclass can bind to CII to trigger activation and thus no Fcγ-receptor IV signalling can take place.

Further detailed investigations on the mode of actions of sialylated IgG's whether at the site of inflammation or systemically (IVIG) are necessary to decipher their regulatory efficacy on the immune system.

The influence of different sialylated IgG subclasses on the development of arthritis remains to be investigated.

5.2.3. Mode of Action

In general, sialylated IgGs might reflect the steady state of a humoral arsenal of antibodies. Upon antigen stimulation, production of desialylated antibodies from new plasma cells might switch the immune system to an inflammatory state to combat pathogens or to trigger autoimmune disease. [21, 23]

There is only little data about the influence of sialylated IgGs on immune cells and only one general regulatory cell signalling pathway has been proposed up to now. [22, 23, 26, 27, 46] SIGN-R1 is a c-lectin receptor on splenic marginal zone macrophages that can bind to sialylated IgGs and triggers the upregulation of inhibitory FcγRIIB on effector macrophages. A switch in the balance from activating to increased inhibitory

signalling upon IgG binding could mark a possible effector mechanism of IVIG, but the human homolog DC-SIGN has a different expression pattern (expressed on dentritic cells) and thus results can not be translated from mouse to human.

In our experiments we could show antiinflammatory effects of sialylated antigen-specific IgG1 antibodies in FcγRIIB$^{-/-}$ mice, strongly suggesting a further interaction pathway contributing to the antiinflammatory activity – especially in an antigen-specific manner. The reduced binding affinity of sialylated IgG1's to the activating FcγRIII and the blocked FcγRIV signalling [9, 20] may account for some of the effects but would not explain active inhibitory modes of action as we observed complete inhibition of the development of severe arthritis compared to the control group even at time-points, when increased IgG1 levels from the treatment could not be detected any more. This would suggest a long lasting regulatory effect of sialylated IgGs to maintain suppressor functions even after natural degradation of the injected biologics.

Interestingly, in healthy and arthritic mice the humoral immune response against chicken CII (chCII) and mouse self-CII (mCII) was at comparable levels. Even healthy or treated mice hat elevated levels of all Ig isotypes and subclasses. Mice treated with sialylated IgG1 had reduced IgG levels at the end of the experiment. These findings are consistent with the suggestion that sialylated IgG1 has a regulatory function and prolonged inflammatory IgG production is reduced. Investigations on the role of Tregs and regulatory macrophages would be of particular interest as well as examinations on the regulatory properties of B-cells.

Healthy and sick mice did significantly differ in their humoral immune response at IgG2a and IgG2b levels, which have been described to have pathogenic properties in autoimmune diseases. [10]

A prognostically relevant observation was that sialylated IgG1 might directly trigger IgM production against self-antigen mCII at early time-points (day 12) – before the onset of the disease (around day 20), which later might prevent the mice from severe arthritis. This correlates with our findings that mice receiving sialylated anti-CII IgG1 before disease onset have a better clinical prognosis and that they have increasee anti-mCII IgM antibodies. One possible mechanism of action of sialylated IgG1 could

thus be the trigger of “natural” IgM production that prevents mice from developing pathological immune responses against self-antigen mCII, because the purpose of IgM is non-inflammatory antigen clearance. [47] Sialylated antibodies might have a direct influence on B-cells, which upon expression of protecting “natural” IgM could have a regulatory role during an immune response.

If certain arthritogenic epitopes on CII account for any effects is unclear. Additionally to B-cell epitopes (antibody epitopes) there are T-cell epitopes on CII [29, 43, 48, 49] that might be sterically blocked upon binding of anti-CII antibodies.
CIA arthritis also evokes a T-cell response during the establishment of inflammation in the joints. [29, 39, 44] But only little is known about the influence of sialylated antibodies on T-cells – especially regulatory T-cells (Tregs) and proinflammatory Th17-cells. [27]

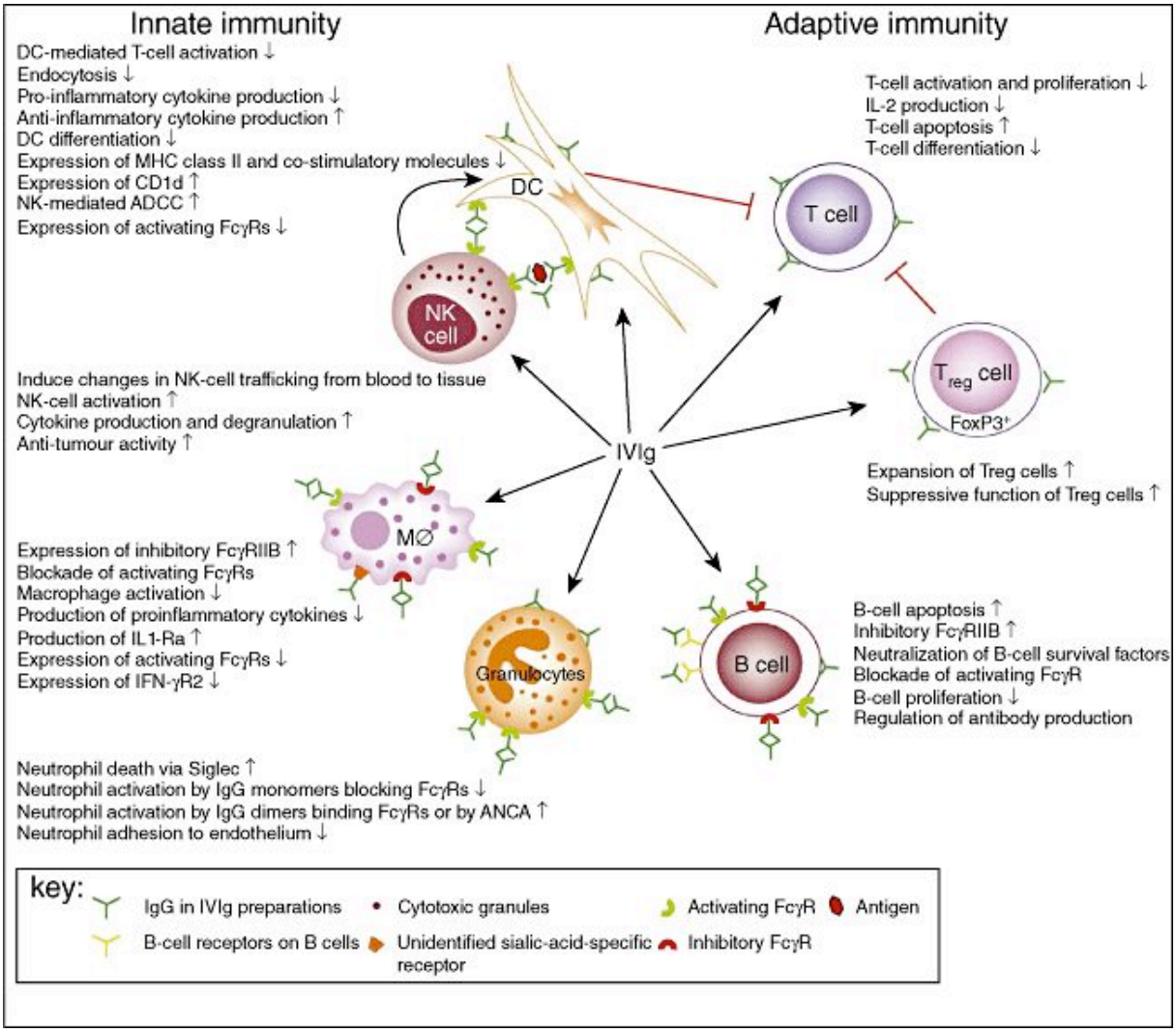

Figure 21 Supposed mechanisms of action of intravenous immunoglobulin (IVIG) on cellular immunity. [23] Besides the many proposed interactions of sialylated IgG's with different cell types of the adaptive and innate immune system depicted in this figure, we suggest novel unidentified modes of action of antigen-specific sialylated IgG antibodies, because our results have excluded the inhibitory Fcγ-receptor IIB as regulatory downstream signal of sialylated IgG's. Up to now there exist several proposed modes of action, but only few publications have suggested direct or indirect interactions and regulation mechanisms of sialylated IgG's with B-cells, T-cells, peripheral macrophages and other components of the immune system.

5.2.4. Perspectives for Medical Applications

Up to now approved antigen-specific antibody biologics have been designed to tag antigenic structures for depletion. In chronic inflammatory autoimmune diseases therapeutic antibodies are used to target proinflammatory cytokines or immune cells. Sialylated IgGs now provide a novel tool for actively transferring tolerance to an

antigenic structure. Translational medicine research in the field of autoimmune diseases will outcome screening methods to individually determine antigenic structures in antibody-mediated autoimmune diseases like rheumatoid arthritis. The design of antigen-specific sialylated antibodies thereafter can be the base of a completely new approach to patient-oriented treatment of multiple autoimmune diseases. Individualized medical therapeutics are necessary to target the causes of disease.

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Appendix

Abbreviations

AA	Acrylamide/bisacrylamide
AgeI	Restriction enzyme
anit-CII-cc	Desialylated anti-collagen type II antibody (cell culture produced)
anti-CII	Anti-collagen type II antibody
anti-CII-sial	Sialylated anti-collagen type II antibody (enzymatic. glycosylated)
Asn	Asparagine
bCII	Bovine collagen type II
BsiWI	Restriction enzyme
C18	Carbohydrate of 18 carbon atoms
C57BL/6	Black-6 mouse strain
CDR	Complementary determining region
CFA	Complete Freund's adjuvants
CFU	Colony forming unit
CH1	Heavy chain constant domain 1
chCII	Chicken collagen type II
CIA	Collagen-induced arthritis
CII	Collagen type II
CL	Light chain constant domain
CMP	Cytidine monophosphate
CO_2	Carbon dioxide
C-region	Constant region
DC-SIGN	Dentritic cell specific ICAM-3 grabbin non-integrin related
ddH_2O	Destilled deionized water
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
dsDNA	Double strand DNA
ELISA	Enzyme-linked immunosorbant assay
EndoS	Endoglycosidase
Fab	Antigen-binding fragmen
Fc	Fragment crystallizable

FCS	Fetal calf serum
FcγRIIB	Fc-gamma receptor II B
G0	Fc-glycan with terminal N-acetylglucosamines
G1	Fc-glycan with one terminal galactose
G2	Fc-glycan with two terminal galactoses
GlcNac	N-acetylglucosamine
H-2^b	MHC haplotype
H_2SO_4	Sulfuric acid
H37Ra	Mycobacterium tuberculosis strain
HC	heavy chin
hCII	Human collagen type II
HCl	Hydrogen chloride
HCMV	Human cytomegalo virus
HEK293T	Human embryonic kidney cells with T-antigen
HindIII	Restriction enzyme
HRP	Horseradish peroxidase
IFA	Incomplete Freund's adjuvants
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
Igκ	Immunoglobulin kappa
IL-1	Interleukin 1
IL-17	Interleukin 17
IVIG	Intravenous immunoglobulin G
kDA	Kilodalton
LB	Lysogeny broth
LC	light chain
LPS	Lipopolysaccharide
M	Molar = mol/L
MALDI-TOF	Matrix-assisted laser desorption/ionization
mCII	Mouse collagen type II
MCS	Multiple cloning site
MHC	Major histocompatibility complex
mM	Millimolar = mmol/L
N-linked	Chemical bound via nitrogen
OD	Optical density
PAD	Peptidyl arginine deaminase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
S1	Fc-glycan with one terminal sialic acid
SalI	Restriction enzyme
SDS	Sodium dodecyl sulfate
SIGN-R1	Specific ICAM-3 grabbin non-integrin related 1

SOC	Super optimal broth
ssDNA	Single strand DNA
St6Gal1	Beta-1,6-sialyltransferase
TAE	Tris acetate EDTA
TLR2	Toll-like receptor 2
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
U	Unit
UDP	Uracile diphosphate
VDJ	Variable-diversity-joining
V_H	Variable region of heavy chain
VJ	Variable-joining
V_L	Variable region of light chain
V-region	Variable region

Curriculum Vitae

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Education

Since 03/2003	Diploma studies in Molecular Biology at the University of Vienna
03/2009 – 10/2009	Diploma student at the German Rheumatism Research Center Berlin (DRFZ)
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Scientific Experiences

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10/2009	Autumn School "Current Concepts in Immunology" congress in Bad Schandau. Oral presentation on "Anti-inflammatory IgG Antibodies in Rheumatoid Arthritis"
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Expertises

Fields	Immunology, Cell Biology and Molecular Medicine
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Studentships

Diploma Thesis	Studentship from the University of Vienna for doing my diploma thesis in Berlin
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Felix Lorenz Vienna, April 2010

Lebenslauf

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10/2009	Herbstschule "Current Concepts in Immunology" Kongress in Bad Schandau. Vortrag über "Anti-inflammatory IgG Antibodies in Rheumatoid Arthritis"
05/2009	Deutsches Tierversuchszertifikat verliehen durch die Teilnahme an einem theorischen und praktischen Mauskurs

08/2008 – 09/2008	Praktikum am Max-Planck-Institut für Infektionsbiologie Berlin in der Gruppe von Dr. Hedda Wardemann - Moleculare Immunologie
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Fachgebiete

Fields	Immunologie, Zellbiologie und Molekulare Medizin Erfahrung im experimentellen Umgang mit Mäusen (z.B. Kollagen-induzierte Arthritis)

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