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Endoglycosidase treatment abrogates IgG arthritogenicity – importance of IgG glycosylation in arthritis

Kutty Selva Nandakumar^{1*}, Mattias Collin², Arne Olsén², Falk Nimmerjahn^{3,5}, Anna M. Blom⁴, Jeffrey V. Ravetch⁵ and Rikard Holmdahl¹

¹Medical Inflammation Research, Lund University, Sweden. ²Division of Infection Medicine, Lund University, Sweden. ³Present address, Experimental Immunology and Immunotherapy, Nikolaus Fiebiger Center for Molecular Medicine, University of Erlangen-Nuremberg, Germany, ⁴Department of Laboratory Medicine, Lund University, Sweden. ⁵Laboratory of Molecular Genetics and Immunology, Rockefeller University, USA.

Key Words: arthritis - IgG – EndoS

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; CII, collagen type II, CAIA, Collagen antibody induced arthritis; LCA, *Lens culinaris* agglutinin

*** Address correspondence**

Dr. Kutty Selva Nandakumar

Medical Inflammation Research

Lund University, BMC, I11, SE-22184, Sweden

Phone: +46-46-222 3349; Fax: +46-46-222 3110; E-mail: nan@med.lu.se

Summary

Glycosylation status of IgG has been implicated in the pathology of rheumatoid arthritis. Earlier, we reported the identification of a novel secreted endo- β -*N*-acetylglucosaminidase (EndoS) secreted by *Streptococcus pyogenes* that specifically hydrolyzes the β -1,4-di-*N*-acetylchitobiose core of the asparagine-linked glycan of human IgG. Here, we analyzed the arthritogenicity of EndoS-treated CII specific mouse mAbs *in vivo*. Endoglycosidase treatment on the antibodies inhibited the induction of arthritis in (BALB/c X B10.Q) F1 mice and induced a milder arthritis in B10.RIII mice as compared with severe arthritis induced by non-treated antibodies. Furthermore, EndoS treatment did not affect the binding of IgG to CII and their ability to activate complement, but it rendered reduced IgG binding to Fc γ Rs and disturbed the formation of stable immune complexes. Hence, the asparagine-linked glycan on IgG plays a crucial role in the development of arthritis.

Introduction

The impact of glycosylation, one of the most important post-translational modifications, on the structure and biological properties of glycoproteins has been well documented [1, 2]. IgG molecules are mainly glycosylated at Asn-297 of the CH2 domain within the Fc region [3, 4] with variable galactosylation but limited sialylation. The remaining glycosylation occurs in the hypervariable regions of the Fab region, with position and frequency of occurrence dependent on the presence of the consensus sequence Asn-Xaa-Thr/Ser for N-glycosylation and is characterized by a high incidence of sialylated structures. Murine IgGs contain 2.3 asparagine-linked (N-linked) biantennary oligosaccharide chains per molecule [5], and human IgGs, 2.8 [6]. The minimal oligosaccharide structure is a hexasaccharide (GlcNAc₂Man₃GlcNAc) with variable sugar residues attached, which results in the generation of the multiple glycoforms. About 30 variants of biantennary chains occur, resulting in many different glycoforms of IgG [6]. X-ray crystal electron density maps of the IgG-Fc revealed that the N297 linked glycan is sequestered within the internal space enclosed by the CH2 domains. There are extensive non-covalent interactions between the carbohydrate and the protein moiety, resulting in reciprocal influences on conformation [3].

These complex biantennary-type oligosaccharides attached to IgG have been shown to be essential for effector functions mediated through Fc receptors (FcRs) and

complement C1q [7, 8]. Furthermore, the Fc glycans of IgG are critically involved in the structural integrity of the antibody [9-11]. Modifications in these oligosaccharides affect susceptibility to proteolytic degradation, clearance rate, antibody-dependent cellular cytotoxicity (ADCC), as well as complement activation apart from binding to FcRs, monocytes, protein G and C1q/C1 [12-17]. However, Fc-glycosylation is not required either for protein A binding [18] or recognition of antigen [19-22]. Recently, de-fucosylation on the N297 -linked glycan in the Fc part of the Ab has been shown to increase ADCC activity indicating the importance of glyco-engineering of Abs for improved clinical efficacy [23]. On the other hand, Fab N-linked glycosylation in the hypervariable regions, while occurring much less frequently, has been reported to influence the binding affinity of antigens [24-26] and may also be involved in IgG self-association, aggregation, and cryo-precipitation [27].

Glycoside hydrolases (EC 3.2.1.-) are a group of enzymes found in all types of organisms including bacteria and mammals, which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Earlier, we reported a novel secreted endo- β -*N*-acetylglucosaminidase, a member of the glycosyl hydrolases of family 18 (FGH18) in *S. pyogenes* (EndoS), which specifically hydrolyzes the β -1,4-di-*N*-acetylchitobiose core of the asparagine-linked glycan of human IgG [28]. EndoS has

similarities to endo- β -*N*-acetylglucosaminidases that cleave the β 1–4 linkage between the two *N*-acetylglucosamines found in the core of the N-linked glycan of IgG. EndoS exclusively hydrolyzes the complex-type biantennary glycan on the heavy γ -chain of native IgG [28]. Endoglycosidase activity on the IgG molecule by EndoS altered its function through impaired Fc γ R binding as well as decreased activation of the classical pathway of complement, which ultimately led to increased bacterial survival in human blood [29].

Antibodies to self-antigens [collagen type II (CII), citrullinated antigens, rheumatoid factors etc.] are prevalent in rheumatoid arthritis (RA) patients and may play a role in arthritis. CII specific mAbs induce an acute form of arthritis in mice, the collagen antibody induced arthritis (CAIA) [30-33] that resembles effector phase of collagen induced arthritis (CIA) without involving priming phase. This antibody-mediated arthritis is dependent on complement components [34], Fc γ Rs [35, 36], effector cytokines TNF- α and IL-1 β [37], and on the cellular players, neutrophils and macrophages [32]. We used CAIA in the present study to understand the importance of glycosylation of IgG molecule by EndoS treatment. Removal of the N-linked glycan on the IgG antibodies rendered them less arthritogenic. Although endoglycosidase activity did not affect the binding of IgG to CII and complement activation, it reduced IgG binding to Fc γ Rs and formation of stable immune complexes.

Results

EndoS removes carbohydrate moieties from CII-specific mouse mAbs

EndoS specifically hydrolyzes the β -1,4-di-N-acetylchitobiose core of the N-linked glycan of IgG, which can be visualized by a size difference (4kDa) on SDS-PAGE and lectin blot analysis using *Lens culinaris* agglutinin (LCA) (Fig 1). LCA recognizes sequences containing α -linked mannose residues and is enhanced in its affinity binding by the core fucose attached to the GlcNAc closest to the asparagine on the protein backbone. LCA lectin blot analysis of the samples revealed a significantly reduced signal when incubated with EndoS. Loss of lectin signal has previously been shown to correspond well to the complete digestion of the chitobiose core of the glycan on N297 of human γ -chains as determined by mass spectroscopy [38]. In contrast, the γ -chains were easily recognized by the LCA lectin when incubated in the absence of EndoS. These data support the hypothesis that EndoS has the ability to remove structures containing α -1,3 mannose from the γ -chains of mouse IgG.

EndoS-treated antibodies bind to cartilage *in vivo*

To understand whether the removal of carbohydrate moieties from CII specific IgG mAbs affected their capacity to bind joint cartilage *in vivo*, we injected mAb into neonatal DA rats. Paw samples collected 24 hours after mAb injection were sectioned and stained with anti-kappa antibodies. There was no difference in the

binding pattern of EndoS treated and untreated antibodies to the joint cartilage *in vivo* demonstrating that the removal of carbohydrates by EndoS did not affect antigen binding capacity of the mAb (Fig 2). Similarly, we did not find any difference in the sera levels of these injected antibodies i.e. clearance of normal and EndoS treated antibodies *in vivo* (data not shown).

Arthritogenicity of anti-CII mAbs is abrogated by endoglycosidase treatment

A cocktail of two CII specific mAbs [M2139 (IgG2b) binding to J1 epitope (551-564; GERGAAGIAGPK) and CIIC1 (IgG2a) binding to C1^I epitope (359-363; ARGLT)] induced an acute arthritis in mice (CAIA) that resembles effector phase of arthritis [39]. To understand whether the removal of carbohydrate side chains affect the arthritis inducing capacity of pathogenic mAbs, we injected a cocktail of mAbs treated or untreated with EndoS. As shown in the Fig. 3, there was a profound inhibition of clinical arthritis in (BALB/c X B10.Q) F1 and B10.RIII mice that received EndoS treated antibodies. These strains of mice were earlier shown to be highly susceptible for CAIA [32]. There was a massive infiltration of cells as well as cartilage and bone erosion in the joints from mice injected with normally glycosylated antibodies. In contrast, mouse paws showed only minor bone erosion and no significant cell infiltration after the transfer of Endo S treated mAbs. Joint articular cartilage from these mice looked normal (Fig. 3). Thus, these results clearly indicate that the removal of the N-linked glycan of IgGs by EndoS abrogates their arthritis inducing capacity in two different genetic backgrounds and as demonstrated

earlier less arthritogenic capacity of the EndoS-treated antibodies was not due to the inability of these antibodies binding to the target antigen.

EndoS-treated mAbs bind C1q and activate complement

The IgG heterosaccharides are known to be involved not only in the stabilization of Fc region binding sites for C1q [19, 40] but also in the structural properties of the Fc region [41]. Therefore, we tested *in vitro*, whether the removal of carbohydrate from γ -chains of IgG by EndoS reduced or abolished the ability of mAbs to activate complement (Fig. 4). We found that there was no difference in the first complement component C1q deposition on mAbs +/- EndoS treatment bound to CII (Fig.4A) or coated directly to plastic surface (Fig.4B). Similarly, there was no difference between treated and non-treated antibodies with regard to deposition of C3b (Fig 4C and D). Interestingly, CIIC1 mAb (+/- EndoS treatment) bound negligible amounts of C1q and did not cause any activation of complement as has been previously reported [34]. However, when the antibodies were directly coated on plates, CIIC1 was able to bind C1q and cause deposition of C3b.

EndoS-treated antibodies bind less efficiently to Fc γ receptors

Since, there was no difference in the complement activation as well as in the ability to bind antigens between fully glycosylated and EndoS-treated antibodies, we next asked the question; why the removal of carbohydrate from γ -chains of IgG reduced/abolished the clinical arthritis? Using SPR (Biacore) kinetic analysis, we

found that EndoS-treated antibodies have lower affinity to recombinant FcγI, FcγIIb, FcγIII and FcγIV proteins (Fig. 5 and Table 1). These findings provide one possible explanation for the loss of arthritogenicity of treated EndoS-treated mAbs because arthritis in CAIA involves FcγR systems apart from activation of complement components.

IgG glycosylation status affects stable immune complex formation

It is most likely that an early step in the initial triggering event in the CAIA model is the binding of the antibodies to CII in the cartilage matrix and the formation of collagen-IgG immune complexes [42, 43]. The CII epitopes recognized by antibodies are located in a repetitive structure formed by CII molecules within the matrix and on the surface of the cartilage [42, 44]. Hence, it is possible that the two different antibodies can form multimeric complexes favoring arthritogenicity either by optimal complement activation or binding to FcγR bearing cells. Similarly, immune complex formation precipitating on the joint surface was found to be absolutely required for arthritis induction in the anti-G6PI serum transfer induced arthritis [45]. Furthermore, Fc-Fc interactions are found to be important for immune complex formation [46-48] and carbohydrates present in the CH2 domain of IgG might have an important role in this process [19]. To determine whether removal of sugar moieties from the CH2 domain of Fc part of IgG could affect the formation of stable immune complexes, single immunodiffusion assay was performed on CII impregnated agarose gel. As shown in the Fig.6 and table 2, EndoS-treated

antibodies did not form stable immune complexes compared to glycosylated mAbs. The inability to form stable immune complexes could be yet another reason for the loss of arthritogenicity of treated antibodies.

Discussion

Carbohydrates present in the CH2 domain of IgG have an important role in its effector functions [19, 40] and also in structural properties of Fc regions [41]. In the present study we found that removal of the N-linked glycan in the CH2 domain of the CII specific IgG mAbs rendered them less arthritogenic in the CAIA mouse model. Although endoglycosidase treatment did not affect the binding of IgG to CII, clearance of antibodies *in vivo*, oxidative burst by neutrophils and macrophages *in vitro* (data not shown) and complement activation, it reduced IgG binding to Fc γ Rs and formation of stable immune complexes.

Earlier studies suggest a pathogenic role for agalactosyl form of IgG in arthritis. Serum IgG from patients with RA and a small number of other rheumatic diseases contains the same set of N-linked bi-antennary oligosaccharides found in normal individuals, although in very different and characteristic amounts [6, 49]. The incidence of structures lacking galactose is dramatically increased in arthritis. Interestingly, elevated levels of agalactosyl glycoforms were found in female RA patients, while decreased levels were correlated with disease remission during gestation followed by postpartum recurrence [50]. The glycoform distribution of

serum IgG was shown to change with age [51, 52]. It has been suggested that low galactosylation of IgG may have a critical role in the pathology of autoimmune disorders such as RA and SLE [53-55], but can also occur through aging [56]. Functional differences have been recognized between these glycoforms. Recently, Nimmerjahn et al [57] have demonstrated that agalactosyl IgG mediates its activity by binding to FcRs but not complement.

On the other hand, endoglycosidase activity on IgG compromises the recognition by all three cellular FcRs [21, 58, 59]. Thermal stability and functionality of the CH2 domains of IgG are progressively reduced with successive removal of outer-arm sugar residues [60]. Aglycosylated IgG fails to activate complement [19], is more liable to proteolytic attack [18] and is not recognized by cells expressing Fc γ RI and II receptors [61]. Furthermore, removal of the complete carbohydrate moiety abolished ability to activate complement and ADCC of a human IgG1 mAb, Campath-1H, but left antigen and protein A binding activity intact, whereas removal of terminal sialic acid residues through glycopeptidase-F digestion did not affect any of the tested IgG activities. [13]. Moreover, sialylated IgG autoantibodies remained poorly pathogenic because of the limited Fc-associated effector functions and loss of cryoglobulin activity [62, 63]. These contradictory observations might be explained by the length and nature of residual carbohydrate structures that remained after cleavage from IgG molecule.

Furthermore, we found no difference in the C1q and C3b deposition on mAbs (+/- EndoS treatment) bound to CII or plastic surface. Interestingly, CII bound CIIC1 (both Endo S treated and untreated) mAb initiated negligible amounts of C1q binding and also did not cause any complement activation as measured by C3b deposition. However, when the antibodies were directly coated on plates, CIIC1 was able to bind C1q and cause deposition of C3b implying that it is the orientation of these antibodies when bound to collagen that precludes binding of C1q and activation of complement. We observed similar phenomenon with different mAbs directed against C1^{III} but not other CII epitopes (data not shown).

Thus, it will be interesting to further analyze the carbohydrate structure-function relationship of IgG molecules using recombinant EndoS for future analytic and therapeutic applications in autoimmune diseases.

Materials and Methods

Animals

The founders of our B10.Q and B10.RIII mice originate from Professor Jan Klein (Tubingen, Germany) stock and has since more than 20 years been maintained in our laboratory. Thus, these strains are named B10.Q/Hd and B10.RIII/Hd. BALB/c mice were obtained from Jackson laboratories (Bar Harbor, ME). (BALB/c x B10.Q) F1 mice shortnamed QB and DA/Han rats were bred in the Medical Inflammation

Research animal house facility in Lund. Four to six month old male mice were used in all the experiments. All the animals were kept in a conventional but barrier animal facility with a climate controlled environment having 12-h light/dark cycles in polystyrene cages containing wood shavings, fed standard rodent chow and water *ad libitum*. Local animal welfare authorities permitted the animal experiments.

Purification of CII specific mAbs

The CII specific hybridomas were generated and characterized as described in detail elsewhere [64, 65]. The anti-CII antibody producing hybridomas, M2139 and CIIC1 were cultured in ultra low bovine IgG containing DMEM Glutamax-I culture medium (Gibco BRL, Invitrogen AB, Sweden) with 100 mg/l of Kanamycin monosulfate (Sigma, St. Louis, USA). MAbs were generated in large scale as culture supernatant using integra cell line 1000 (CL-1000) flasks (Integra Biosciences, Switzerland). Antibodies were purified using γ -bind plus affinity gel matrix (GE Healthcare, Sweden) and Äkta purification system (GE Healthcare, Sweden). Briefly, culture supernatants were centrifuged at 12500 rpm for 30 min, filtered and degassed before applying to the gel matrix. The gel was washed extensively and the antibodies were eluted using acetic acid buffer at pH 3.0 and neutralized with 1 M Tris-HCl, pH 9.0. The peak fractions were pooled and dialyzed extensively against PBS, pH 7.0 with or without azide. The IgG content was determined by freeze-drying. The antibody solutions were filter sterilized using 0.2 μ m syringe filters (Dynagard, Spectrum Laboratories, CA, USA), aliquoted and stored at -70°C until

used. Amount of endotoxin content in the antibody solutions prepared was found to be negligible and the antibodies induced arthritis in the TLR-4-deficient (LPS non-responder) mice [32].

EndoS-treatment of mAbs

MAbs C1 and M2139 were hydrolyzed with recombinant EndoS fused to GST (GST-EndoS) purified as previously described [66] Enzyme/substrate molar ratio was 1:400 in PBS and samples were incubated for 24h at 37°C. GST-EndoS was removed from the samples by passing three times over a glutathione-Sepharose column (Amersham Biosciences, Uppsala, Sweden). One μg of treated and untreated IgG was separated by 10% SDS-PAGE followed by staining with Coomassie Blue. For lectin blot analysis, 100 ng of IgG was separated as above and blotted to Immobilon-P PVDF-membrane (Millipore, Bedford, MA). Membranes were blocked with 10 mM HEPES (pH 7.5) with 0.15 M NaCl, 0.01 mM MnCl_2 , 0.1 mM CaCl_2 , and 0.1% Tween-20 (HBST) and incubated with 1 $\mu\text{g}/\text{ml}$ of biotinylated *Lens culinaris* agglutinin (LCA) lectin (Vector Laboratories, Burlingame, CA). After washing in HBST, membranes were incubated with 50 ng/ml of peroxidase-labeled streptavidin (Vector Laboratories). After washing, membranes were developed using Super Signal West Pico Chemiluminiscent Substrate (Pierce, Rockford, IL) and developed using a ChemiDoc XRS imaging system (BioRad, Hercules, CA).

Complement assays

All incubation steps were made with 50 µl solution for 1 h and in room temperature except when stated otherwise. Every step was followed by extensive washing with 50 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween; pH 7.5. Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated over night at 4°C with either CII (10 µg/ml) or directly with the mAbs diluted in 75 mM Na-carbonate, pH 9.6. The wells were blocked for 2 h with 200 ml of 3 % fish gelatine in washing buffer (blocking buffer). The plates coated with CII were incubated for with 10 µg/ml of each antibody diluted in the blocking buffer and washed. Dilutions of (BALB/c x B10.Q) F1 serum in DGVB⁺⁺ (2.5 mM veronal buffer pH 7.3, 70 mM NaCl, 140 mM glucose, 0.1 % gelatine, 1 mM MgCl₂ and 0.15 mM CaCl₂) were added to the plates and incubated for 1h at 37°C, followed by incubation with specific digoxigenin-labelled rat polyclonal antibodies against mouse C1q (generous gift of professor Daha and Dr Trouw, Leiden University) or FITC-labelled goat anti-mouse C3 antibodies (ICN Biomedicals/Cappel, Aurora, OH), both diluted 1:1000 in blocking solution. Horseradish peroxidase (HRP)-labeled secondary antibodies against goat Igs (Dako, Glostrup, Denmark) or digoxigenin (Roche Applied Science, Indianapolis, USA) were then allowed to bind (both diluted 1:1000 in the blocking buffer). Bound enzyme was assayed using 1,2-phenylenediamine dihydrochloride (OPD) -tablets (Dako) and absorbance was measured at 490 nm.

Surface plasmon resonance (SPR) analysis

SPR analysis was performed as described earlier [67]. Briefly, EndoS treated and untreated mAbs were immobilized on the surface of CM5 sensor chips. Soluble Fc γ -receptors were injected at 5 different concentrations through flow cells at room temperature in HBS-EP running buffer (10mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) at a flow rate of 30ml/min. Soluble Fc-receptors were injected for 3 min and dissociation of bound molecules was observed for 7 min. Background binding to control flow cells was subtracted automatically. Control experiments were performed to exclude mass transport limitations. Affinity constants were derived from sensorgram data using simultaneous fitting to the association and dissociation phases and global fitting to all curves in the set. A 1:1 Langmuir binding model closely fitted the observed sensorgram data and was used in all experiments.

Collagen antibody induced arthritis (CAIA)

The two arthritogenic mAbs combination described earlier [32] was used in this study with or without EndoS treatment: M2139 (γ 2b) and CIIC1 (γ 2a) binding to J1 (triple helical MP*GERGAAGIAGPK - P* indicates hydroxyproline) and C1^I (triple helical GARGLT) epitopes. The cocktail of the mAbs (9 mg per mouse) was prepared by mixing equal concentrations of each of the sterile filtered antibody solutions. Mice were injected i.v. with 250-500 μ l solution. As internal controls, mice received equal volumes of PBS. On day 5, all the mice received LPS (50

µg/mice/i.p.). None of the control mice receiving PBS with or without LPS developed arthritis.

Clinical evaluation of arthritis

Mice were examined daily for the arthritis development for a minimum of 21 days or until the inflammation subsided. Scoring of animals was done blindly using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness as described previously [68]. Scoring was recorded in the phalangeal joints (maximum of 1 point per digit, 5 points per paw), the metacarpus or metatarsus (5 points), and in the wrist and ankle joints (5 points). Thus, the maximum score was 15/paw resulting in a peak of 60 for the total joint count.

Histological Preparations

Paws were dissected on the indicated day from each group of mice (3-4 mice per group), fixed in 4% phosphate buffered paraformaldehyde solution (pH 7.0) for 24 hours, decalcified for 3-4 weeks in a solution containing EDTA, polyvinylpyrrolidone and Tris-HCl, pH 6.95 followed by dehydration and embedding in paraffin. Sections of 6 µm were stained with hematoxylin-eosin to determine cellular infiltration and bone and cartilage morphology. For immunohistochemistry, paws were immediately frozen in OCT compound using isopentane on dry ice. The samples were stored at -70°C until cryosectioned at 10

μm at -30°C . Biotinylated rat anti-mouse Ig κ mAb (clone 187.1), streptavidin peroxidase and diaminobenzidine were used for detection of cartilage bound anti-CII antibodies.

Statistical analyses

All the mice were included for calculation of arthritis susceptibility and severity. The severity of arthritis was analyzed by Mann Whitney U test and the incidence by Chi Square test using Statview (version 5.0.1).

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Conflict of interest: Hansa Medical AB has filed patent applications on EndoS and AO, MC, RH, KSN are listed as inventors and the applications are pending.

References

- 1 **Jefferis, R.**, Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 2005. **21**: 11-16.
- 2 **Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. and Dwek, R. A.**, The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* 2007. **25**: 21-50.
- 3 **Deisenhofer, J.**, Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* 1981. **20**: 2361-2370.
- 4 **Sutton, B. J. and Phillips, D. C.**, The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. *Biochem Soc Trans* 1983. **11**: 130-132.
- 5 **Mizuochi, T., Hamako, J. and Titani, K.**, Structures of the sugar chains of mouse immunoglobulin G. *Arch Biochem Biophys* 1987. **257**: 387-394.

- 6 **Parekh, R. B., Dwek, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, T., Matsuta, K. and et al.,** Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 1985. **316**: 452-457.
- 7 **Burton, D. R. and Woof, J. M.,** Human antibody effector function. *Adv Immunol* 1992. **51**: 1-84.
- 8 **Jefferis, R., Lund, J. and Pound, J. D.,** IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol Rev* 1998. **163**: 59-76.
- 9 **Matsuda, H., Nakamura, S., Ichikawa, Y., Kozai, K., Takano, R., Nose, M., Endo, S., Nishimura, Y. and Arata, Y.,** Proton nuclear magnetic resonance studies of the structure of the Fc fragment of human immunoglobulin G1: comparisons of native and recombinant proteins. *Mol Immunol* 1990. **27**: 571-579.
- 10 **Malhotra, R., Wormald, M. R., Rudd, P. M., Fischer, P. B., Dwek, R. A. and Sim, R. B.,** Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat Med* 1995. **1**: 237-243.
- 11 **Wormald, M. R., Rudd, P. M., Harvey, D. J., Chang, S. C., Scragg, I. G. and Dwek, R. A.,** Variations in oligosaccharide-protein interactions in

immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* 1997. **36**: 1370-1380.

- 12 **Bond, A., Jones, M. G. and Hay, F. C.,** Human IgG preparations isolated by ion-exchange or protein G affinity chromatography differ in their glycosylation profiles. *J Immunol Methods* 1993. **166**: 27-33.
- 13 **Boyd, P. N., Lines, A. C. and Patel, A. K.,** The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. *Mol Immunol* 1995. **32**: 1311-1318.
- 14 **Jefferis, R., Lund, J. and Goodall, M.,** Recognition sites on human IgG for Fc gamma receptors: the role of glycosylation. *Immunol Lett* 1995. **44**: 111-117.
- 15 **Lund, J., Takahashi, N., Goodall, M., Pound, J. D. and Jefferis, R.,** Oligosaccharide-protein interactions in IgG antibody molecules: structural and functional consequences. *Biochem Soc Trans* 1995. **23**: 102S.
- 16 **Lund, J., Takahashi, N., Pound, J. D., Goodall, M. and Jefferis, R.,** Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fc gamma receptor I and influence the synthesis of its oligosaccharide chains. *J Immunol* 1996. **157**: 4963-4969.

- 17 **Wright, A. and Morrison, S. L.**, Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotechnol* 1997. **15**: 26-32.
- 18 **Leatherbarrow, R. J. and Dwek, R. A.**, The effect of aglycosylation on the binding of mouse IgG to staphylococcal protein A. *FEBS Lett* 1983. **164**: 227-230.
- 19 **Nose, M. and Wigzell, H.**, Biological significance of carbohydrate chains on monoclonal antibodies. *Proc Natl Acad Sci U S A* 1983. **80**: 6632-6636.
- 20 **Leatherbarrow, R. J., Rademacher, T. W., Dwek, R. A., Woof, J. M., Clark, A., Burton, D. R., Richardson, N. and Feinstein, A.**, Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. *Mol Immunol* 1985. **22**: 407-415.
- 21 **Tao, M. H. and Morrison, S. L.**, Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol* 1989. **143**: 2595-2601.
- 22 **Tsuchiya, N., Endo, T., Matsuta, K., Yoshinoya, S., Aikawa, T., Kosuge, E., Takeuchi, F., Miyamoto, T. and Kobata, A.**, Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG. *J Rheumatol* 1989. **16**: 285-290.

- 23 **Nechansky, A., Schuster, M., Jost, W., Siegl, P., Wiederikum, S., Gorr, G. and Kircheis, R.,** Compensation of endogenous IgG mediated inhibition of antibody-dependent cellular cytotoxicity by glyco-engineering of therapeutic antibodies. *Mol Immunol* 2007. **44**: 1815-1817.
- 24 **Wright, A. and Morrison, S. L.,** Antibody variable region glycosylation: biochemical and clinical effects. *Springer Semin Immunopathol* 1993. **15**: 259-273.
- 25 **Endo, T., Wright, A., Morrison, S. L. and Kobata, A.,** Glycosylation of the variable region of immunoglobulin G--site specific maturation of the sugar chains. *Mol Immunol* 1995. **32**: 931-940.
- 26 **Leibiger, H., Wustner, D., Stigler, R. D. and Marx, U.,** Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. *Biochem J* 1999. **338 (Pt 2)**: 529-538.
- 27 **Parekh, R. B., Roitt, I. M., Isenberg, D. A., Dwek, R. A., Ansell, B. M. and Rademacher, T. W.,** Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity. *Lancet* 1988. **1**: 966-969.
- 28 **Collin, M. and Olsen, A.,** EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *EMBO J* 2001. **20**: 3046-3055.

- 29 **Collin, M., Svensson, M. D., Sjöholm, A. G., Jensenius, J. C., Sjöbring, U. and Olsen, A.,** EndoS and SpeB from *Streptococcus pyogenes* inhibit immunoglobulin-mediated opsonophagocytosis. *Infect Immun* 2002. **70**: 6646-6651.
- 30 **Terato, K., Hasty, K. A., Reife, R. A., Cremer, M. A., Kang, A. H. and Stuart, J. M.,** Induction of arthritis with monoclonal antibodies to collagen. *J Immunol* 1992. **148**: 2103-2108.
- 31 **Terato, K., Harper, D. S., Griffiths, M. M., Hasty, D. L., Ye, X. J., Cremer, M. A. and Seyer, J. M.,** Collagen-induced arthritis in mice: synergistic effect of *E. coli* lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 1995. **22**: 137-147.
- 32 **Nandakumar, K. S., Svensson, L. and Holmdahl, R.,** Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol* 2003. **163**: 1827-1837.
- 33 **Nandakumar, K. S. and Holmdahl, R.,** Efficient promotion of collagen antibody induced arthritis (CAIA) using four monoclonal antibodies specific for the major epitopes recognized in both collagen induced arthritis and rheumatoid arthritis. *J Immunol Methods* 2005. **304**: 126-136.

- 34 **Hietala, M. A., Nandakumar, K. S., Persson, L., Fahlen, S., Holmdahl, R. and Pekna, M.,** Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. *Eur J Immunol* 2004. **34**: 1208-1216.
- 35 **Kagari, T., Tanaka, D., Doi, H. and Shimozato, T.,** Essential role of Fc gamma receptors in anti-type II collagen antibody-induced arthritis. *J Immunol* 2003. **170**: 4318-4324.
- 36 **Nandakumar, K. S., Andren, M., Martinsson, P., Bajtner, E., Hellstrom, S., Holmdahl, R. and Kleinau, S.,** Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcgammaRIIB. *Eur J Immunol* 2003. **33**: 2269-2277.
- 37 **Kagari, T., Doi, H. and Shimozato, T.,** The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL- 6, in the development of monoclonal antibody-induced arthritis. *J Immunol* 2002. **169**: 1459-1466.
- 38 **Collin, M. and Fischetti, V. A.,** A novel secreted endoglycosidase from *Enterococcus faecalis* with activity on human immunoglobulin G and ribonuclease B. *J Biol Chem* 2004. **279**: 22558-22570.
- 39 **Nandakumar, K. S. and Holmdahl, R.,** Antibody-induced arthritis: disease mechanisms and genes involved at the effector phase of arthritis. *Arthritis Res Ther* 2007. **8**: 223-233.

- 40 **Winkelhake, J. L., Kunicki, T. J., Elcombe, B. M. and Aster, R. H.,**
Effects of pH treatments and deglycosylation of rabbit immunoglobulin G on
the binding of C1q. *J Biol Chem* 1980. **255**: 2822-2828.
- 41 **Taves, C. J., Kusumi, A. and Winkelhake, J. L.,** Human aglycosyl-IgG
exhibits increased hydrophobicity. Binding/fluorescence studies with 8-
anilino-naphthalene-1-sulfonic acid (ANS). *Biochem Biophys Res Commun*
1984. **124**: 605-613.
- 42 **Holmdahl, R., Mo, J. A., Jonsson, R., Karlstrom, K. and Scheynius, A.,**
Multiple epitopes on cartilage type II collagen are accessible for antibody
binding in vivo. *Autoimmunity* 1991. **10**: 27-34.
- 43 **Amirahmadi, S. F., Whittingham, S., Crombie, D. E., Nandakumar, K.**
S., Holmdahl, R., Mackay, I. R., van Damme, M. P. and Rowley, M. J.,
Arthritogenic anti-type II collagen antibodies are pathogenic for cartilage-
derived chondrocytes independent of inflammatory cells. *Arthritis Rheum*
2005. **52**: 1897-1906.
- 44 **Carlsen, S., Nandakumar, K. S. and Holmdahl, R.,** Type IX collagen
deficiency enhances the binding of cartilage-specific antibodies and arthritis
severity. *Arthritis Res Ther* 2006. **8**: R102-R109.
- 45 **Wipke, B. T., Wang, Z., Nagengast, W., Reichert, D. E. and Allen, P. M.,**
Staging the initiation of autoantibody-induced arthritis: a critical role for
immune complexes. *J Immunol* 2004. **172**: 7694-7702.

- 46 **Moller, N. P. and Steensgaard, J.**, Fc-mediated immune precipitation. II. Analysis of precipitating immune complexes by rate-zonal ultracentrifugation. *Immunology* 1979. **38**: 641-648.
- 47 **Moller, N. P. and Christiansen, G.**, Fc-mediated immune precipitation. III. Visualization by electron microscopy. *Immunology* 1983. **48**: 469-476.
- 48 **Easterbrook-Smith, S. B., Vandenberg, R. J. and Alden, J. R.**, The role of Fc:Fc interactions in insoluble immune complex formation and complement activation. *Mol Immunol* 1988. **25**: 1331-1337.
- 49 **Parekh, R., Isenberg, D., Rook, G., Roitt, I., Dwek, R. and Rademacher, T.**, A comparative analysis of disease-associated changes in the galactosylation of serum IgG. *J Autoimmun* 1989. **2**: 101-114.
- 50 **Rook, G. A., Steele, J., Brealey, R., Whyte, A., Isenberg, D., Sumar, N., Nelson, J. L., Bodman, K. B., Young, A., Roitt, I. M. and et al.**, Changes in IgG glycoform levels are associated with remission of arthritis during pregnancy. *J Autoimmun* 1991. **4**: 779-794.
- 51 **Parekh, R., Roitt, I., Isenberg, D., Dwek, R. and Rademacher, T.**, Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. *J Exp Med* 1988. **167**: 1731-1736.
- 52 **Yamada, E., Tsukamoto, Y., Sasaki, R., Yagyu, K. and Takahashi, N.**, Structural changes of immunoglobulin G oligosaccharides with age in healthy human serum. *Glycoconj J* 1997. **14**: 401-405.

- 53 **Kuroki, A., Shibata, T., Honda, H., Totsuka, D., Kobayashi, K. and Sugisaki, T.,** Glomerular and serum IgG subclasses in diffuse proliferative lupus nephritis, membranous lupus nephritis, and idiopathic membranous nephropathy. *Intern Med* 2002. **41**: 936-942.
- 54 **Kuroda, Y., Nakata, M., Nose, M., Kojima, N. and Mizuochi, T.,** Abnormal IgG galactosylation and arthritis in MRL-Fas(lpr) or MRL-FasL(gld) mice are under the control of the MRL genetic background. *FEBS Lett* 2001. **507**: 210-214.
- 55 **Kuroda, Y., Nakata, M., Hirose, S., Shirai, T., Iwamoto, M., Izui, S., Kojima, N. and Mizuochi, T.,** Abnormal IgG galactosylation in MRL-lpr/lpr mice: pathogenic role in the development of arthritis. *Pathol Int* 2001. **51**: 909-915.
- 56 **Shikata, K., Yasuda, T., Takeuchi, F., Konishi, T., Nakata, M. and Mizuochi, T.,** Structural changes in the oligosaccharide moiety of human IgG with aging. *Glycoconj J* 1998. **15**: 683-689.
- 57 **Nimmerjahn, F., Anthony, R. M. and Ravetch, J. V.,** Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. *Proc Natl Acad Sci U S A* 2007. **104**: 8433-8437.
- 58 **Sarmay, G., Lund, J., Rozsnyay, Z., Gergely, J. and Jefferis, R.,** Mapping and comparison of the interaction sites on the Fc region of IgG responsible for triggering antibody dependent cellular cytotoxicity (ADCC) through

- different types of human Fc gamma receptor. *Mol Immunol* 1992. **29**: 633-639.
- 59 **Pound, J. D., Lund, J. and Jefferis, R.,** Aglycosylated chimaeric human IgG3 can trigger the human phagocyte respiratory burst. *Mol Immunol* 1993. **30**: 233-241.
- 60 **Mimura, Y., Church, S., Ghirlando, R., Ashton, P. R., Dong, S., Goodall, M., Lund, J. and Jefferis, R.,** The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol Immunol* 2000. **37**: 697-706.
- 61 **Walker, M. R., Lee, J. and Jefferis, R.,** Immunogenicity and antigenicity of immunoglobulins: detection of human immunoglobulin light-chain carbohydrate, using concanavalin A. *Biochim Biophys Acta* 1987. **915**: 314-320.
- 62 **Kaneko, Y., Nimmerjahn, F. and Ravetch, J. V.,** Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 2006. **313**: 670-673.
- 63 **Baudino, L., Azeredo da Silveira, S., Nakata, M. and Izui, S.,** Molecular and cellular basis for pathogenicity of autoantibodies: lessons from murine monoclonal autoantibodies. *Springer Semin Immunopathol* 2006. **28**: 175-184.

- 64 **Holmdahl, R., Rubin, K., Klareskog, L., Larsson, E. and Wigzell, H.,** Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. *Arthritis Rheum* 1986. **29**: 400-410.
- 65 **Schulte, S., Unger, C., Mo, J. A., Wendler, O., Bauer, E., Frischholz, S., von der Mark, K., Kalden, J. R., Holmdahl, R. and Burkhardt, H.,** Arthritis-related B cell epitopes in collagen II are conformation-dependent and sterically privileged in accessible sites of cartilage collagen fibrils. *J Biol Chem* 1998. **273**: 1551-1561.
- 66 **Collin, M. and Olsen, A.,** Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect Immun* 2001. **69**: 7187-7189.
- 67 **Nimmerjahn, F., Bruhns, P., Horiuchi, K. and Ravetch, J. V.,** Fcγ₄R: a novel FcR with distinct IgG subclass specificity. *Immunity* 2005. **23**: 41-51.
- 68 **Holmdahl, R., Carlsen, S, Mikulowska, A, Vestberg, M, Brunsberg, U, Hansson, A-S, Sundvall, M, Jansson, L, and Pettersson, U,** Genetic analysis of murine models for rheumatoid arthritis. In **Adolpho, K.** (Ed.) *Human Genome Methods*. CRC press, New York 1998, pp 215-238.

Table 1: Affinity constants* using surface plasmon resonance (SPR) analysis

mAb	FcγRI	FcγRIIB	FcγRIII	FcγRIV
CIIC1 (IgG2a)	4.32×10^7	0.24×10^6	0.29×10^6	1.86×10^7
CIIC1D	3.71×10^6	$\sim 10^4$	$\ll 10^4$	0.9×10^6
M2139 (IgG2b)	ND	1.87×10^6	0.95×10^6	2.59×10^7
M2139D	ND	0.05×10^6	0.06×10^6	0.54×10^6

*Calculated affinity constants of normal and EndoS treated (D) CII-specific mAbs to four different recombinant FcγRs as measured using Biacore are shown. Affinity constants were derived from sensorgram data using simultaneous fitting to the association and dissociation phases and global fitting to all curves in the set. ND-not done.

Table 2: Quantitative analysis of single immuno-diffusion

mAb	EndoS treatment	Volume x10 ⁴ intensity units/mm ²	Circle area x10 ² mm ²	Circle width in mm	Pattern
CIIC1	-	72.9	76.9	99.1	solid
CIIC1	+	8.7	19.3	49.7	diffused
M2139	-	65.9	67.3	92.8	solid
M2139	+	43.0	47.5	78.0	diffused
IgG1*	-	23.4	27.1	58.9	diffused
IgG2a*	-	15.2	25.8	57.5	partial
IgG2b*	-	1.1	10.3	36.3	diffused

* Control antibodies binding to unrelated antigens (IgG1-ovalbumin; IgG2a-human HLA-DR α ; IgG2b - human parathyroid epithelial cells). Background intensity values were subtracted from actual values. Quantitative measurements of the precipitin circles were done using Quantity One[®] software program (Bio-Rad laboratories, CA, USA).

Figure Legends

Figure 1: Analysis of EndoS treated CII- specific mAbs. SDS-PAGE and lectin blot analysis of IgG mAbs (M2139 – IgG2b and CIIC1 –IgG2a) incubated with and without EndoS and separated by 10% SDS-PAGE. The proteins were detected by Coomassie Brilliant Blue staining (Stain) or by blotting to a membrane that was probed with LCA lectin (Blot).

Figure 2: EndoS-treated antibodies bind to cartilage *in vivo*. One to two days old neonatal rats were injected with 1 mg of CII binding antibody (both normal and EndoS treated [D]) i.p. Twenty-four hours after the antibody transfer, paws were dissected and snap frozen in OCT compound using isopentane and dry ice. Immunohistochemical analysis was performed using biotinylated anti-mouse kappa (187.1) antibody and streptavidin peroxidase as detecting system using standard protocol. Stained joint sections (10 µm) from neonatal rats injected with M2139 (a), M2139D (b), CIIC1(c), CIIC1D (d) and control, untreated rats (e) are shown. (Magnification X 10).

Figure 3: Loss of arthritogenicity by EndoS treated anti-CII mAbs. Groups of male (BALB/c X B10.Q) F1 mice were injected with 9 mg of either untreated (n=7) or EndoS treated (n=5) anti-CII mAbs (M2139 + CIIC1) on day 0. All the mice were injected with 50 µg of *E.coli* LPS i.p. on day 5. Mean arthritis score (A) is shown.

Similarly, male B10.RIII mice were injected with 9 mg of either untreated (n=11) or EndoS treated (n=12) anti-CII mAbs (M2139 + CIIC1) on day 0. All the mice were injected with 50 μ g of *E.coli* LPS i.p. on day 5. Mean arthritis score (B) is shown. All the mice were included for calculations. Error bars indicate mean \pm SEM. **Insert:** Representative figures of ankle joints of (BALB/c x B10.Q) F1 mice (n=3-4) injected with 9 mg of untreated (C) and EndoS treated (D) antibody cocktail. Magnification (10 X).

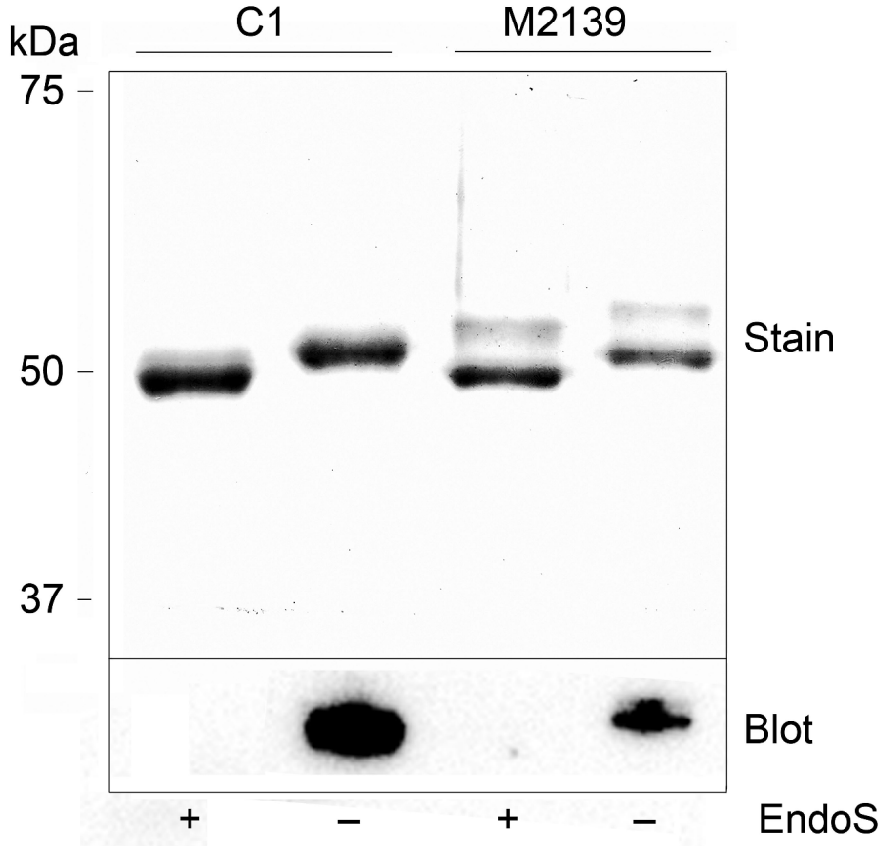
Figure 4: No difference in complement activation (C1q and C3b deposition) of EndoS treated [D] and untreated CII reactive mAbs *in vitro*. Deposition of C1q or C3b from mouse sera on plates coated with CII followed by mAbs using different concentrations of normal (BALB/c x B10.Q) F1 serum (A and B). Deposition of C1q and C3b on antibodies directly coated on plates and incubated with 0.25% or 0.125% normal (BALB/c x B10.Q) F1 serum (C and D). CIIC1 antibodies did not activate complement when bound to collagen type II. Results of three independent determinations are shown as means \pm SD. G11 (IgG2b) and L243 (IgG2a) are control mAbs binding to irrelevant antigens.

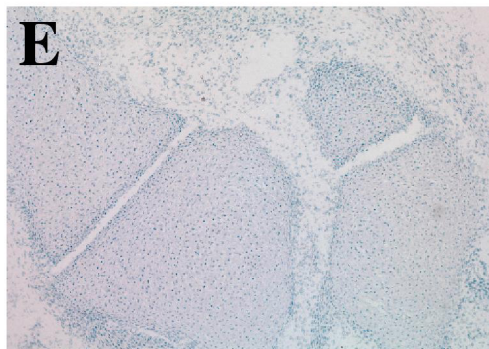
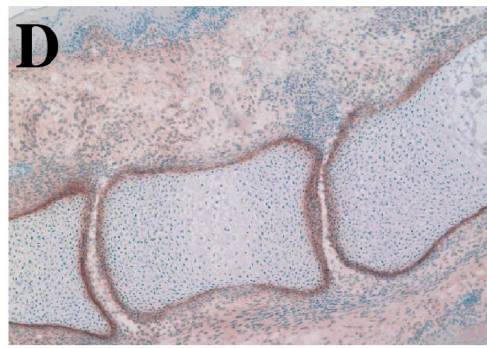
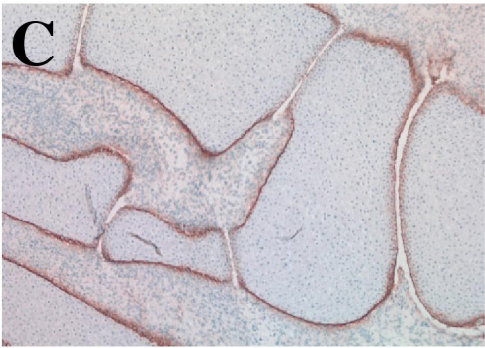
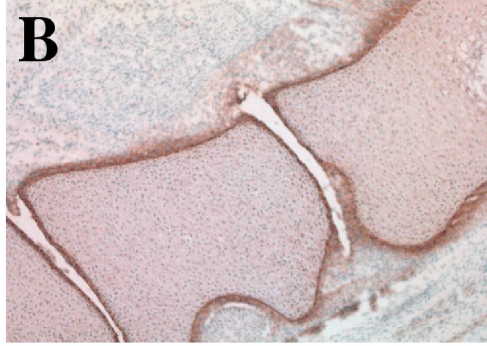
Figure 5: Biacore analysis of affinity between Fc γ R (s) and anti-CII mAbs. Affinity of antibodies to recombinant Fc γ R (I, IIb, III and IV) molecules were determined using Biacore. Shown are the sensorgrams of 5 different concentrations

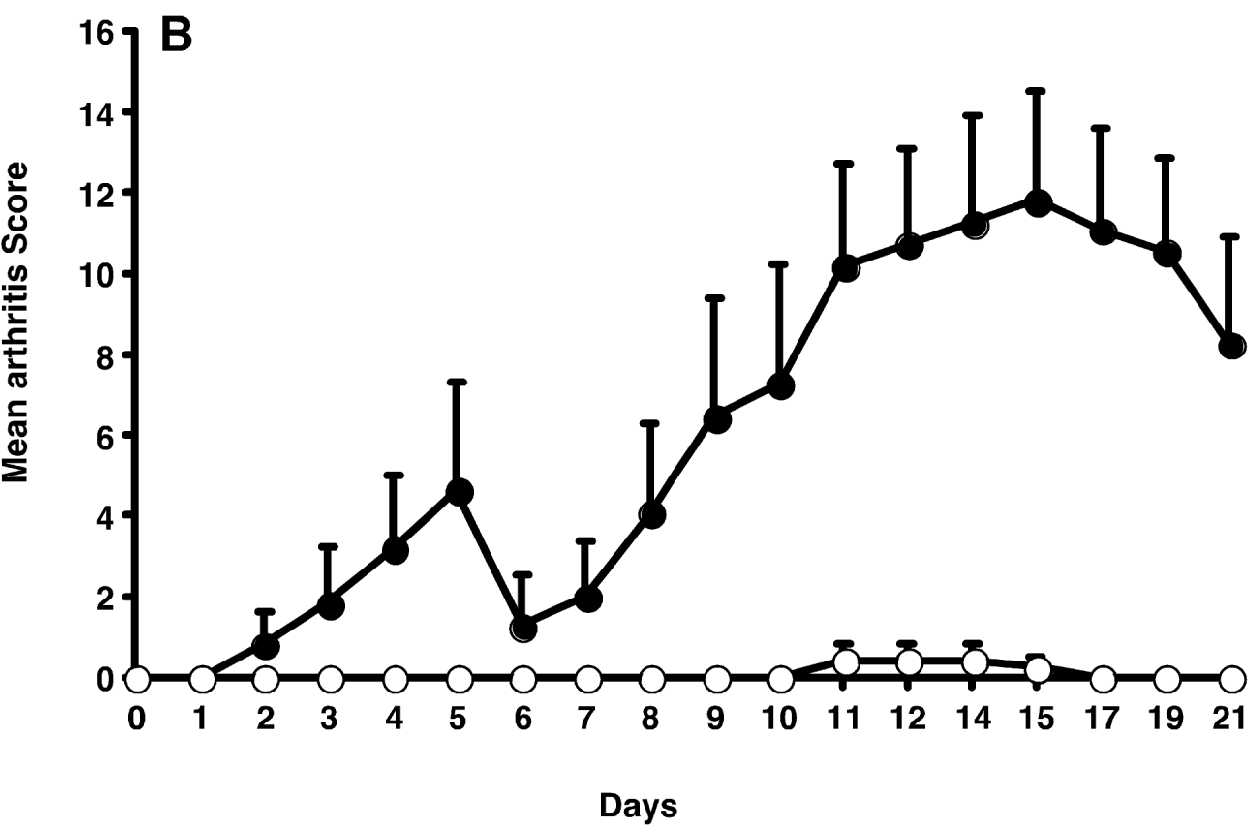
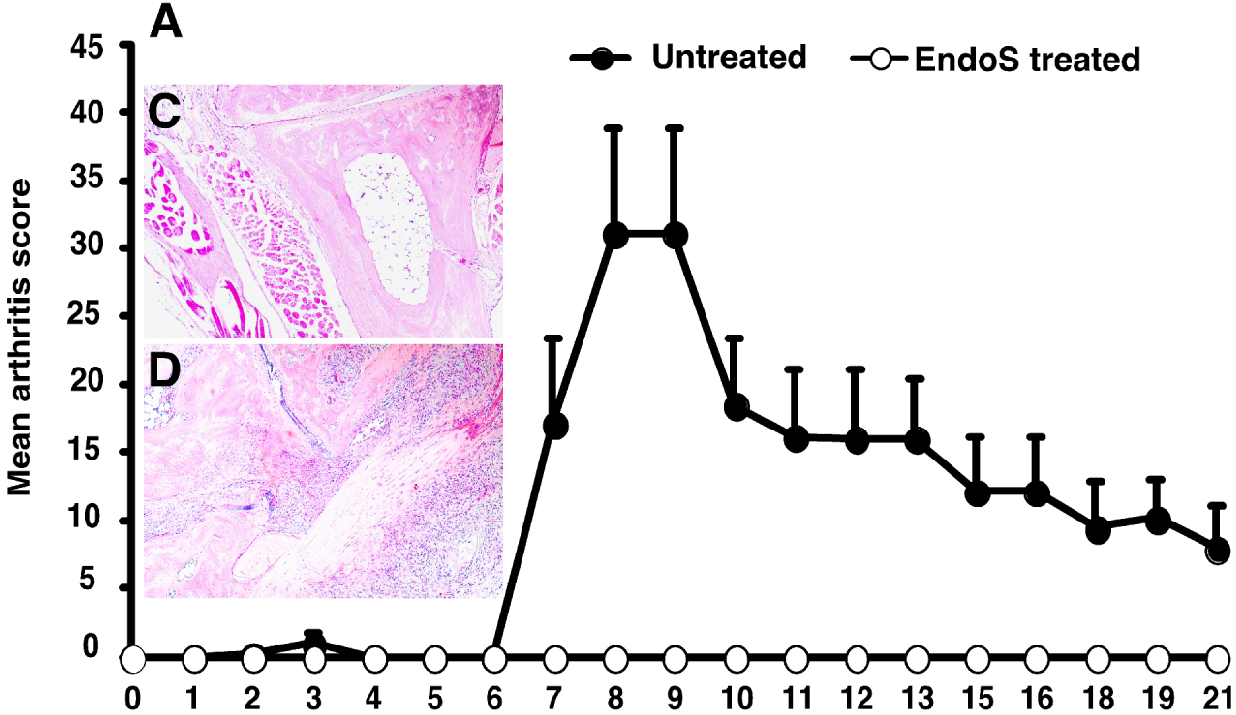
of the four different soluble FcRs to CIIC1-IgG2a and the EndoS treated variant CIIC1D. See Materials and Methods for experimental details.

Figure 6: EndoS-treated antibodies did not form stable immune complexes.

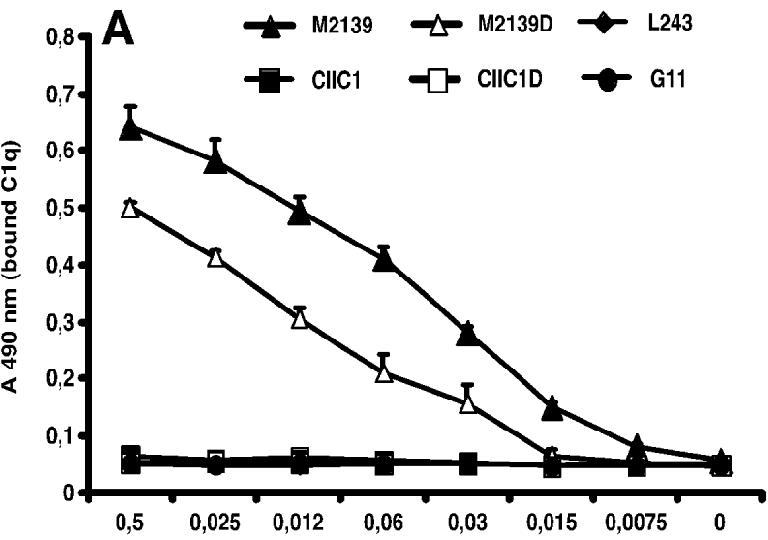
Single immunodiffusion of antibodies in agarose gel. Rat-CII was impregnated in the 1% agarose (low gelling temp. agarose 26-30°C) gel at 1mg/ml in PBS containing 0.05% sodium azide. 25 µl of antibodies at 1mg/ml concentration were loaded per well and incubated at room temperature in a moist chamber for 24 h. Gel was stained with Coomassie Brilliant Blue. EndoS treated antibodies are indicated as deglyco (D).



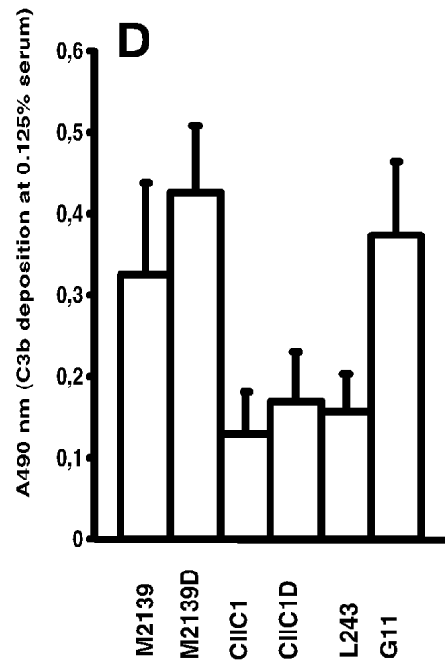
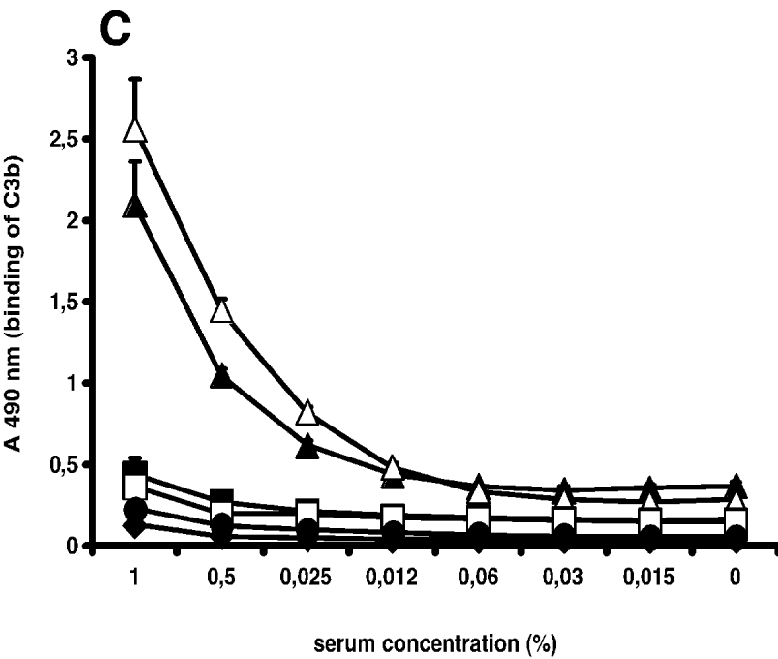
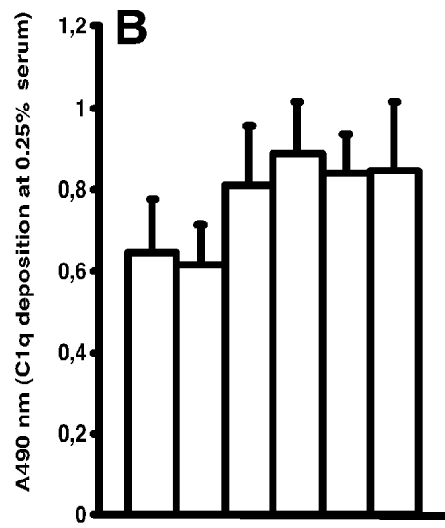


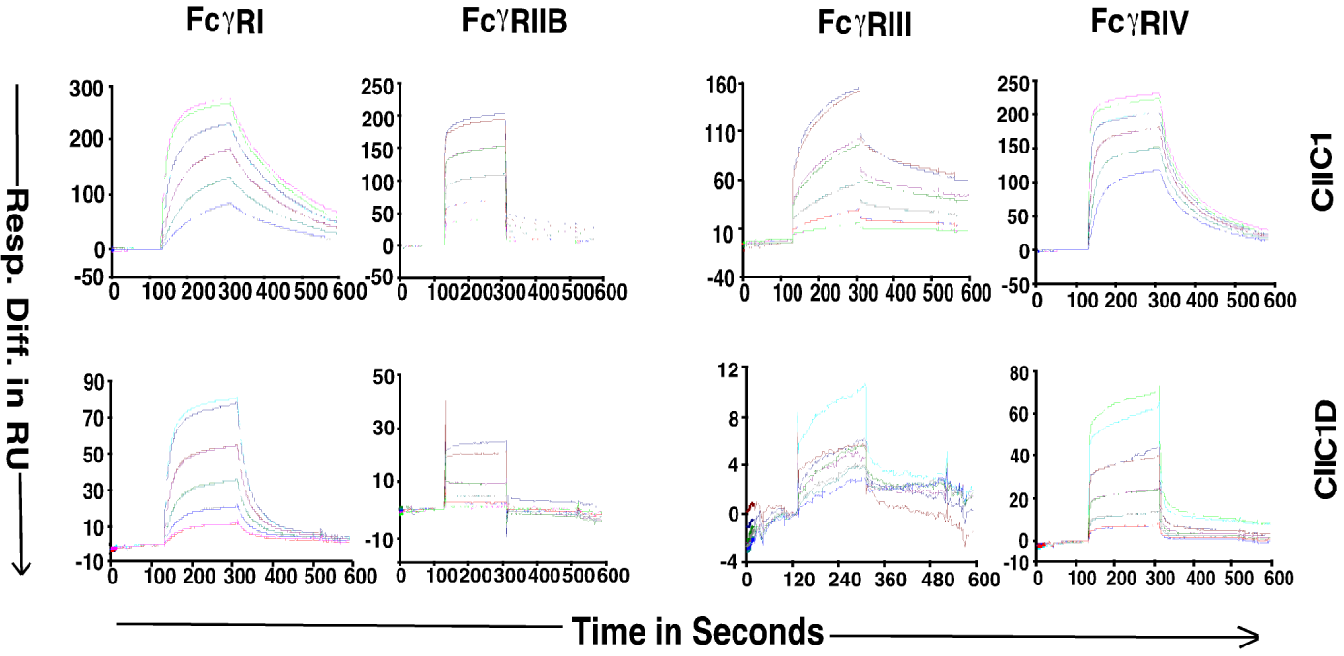


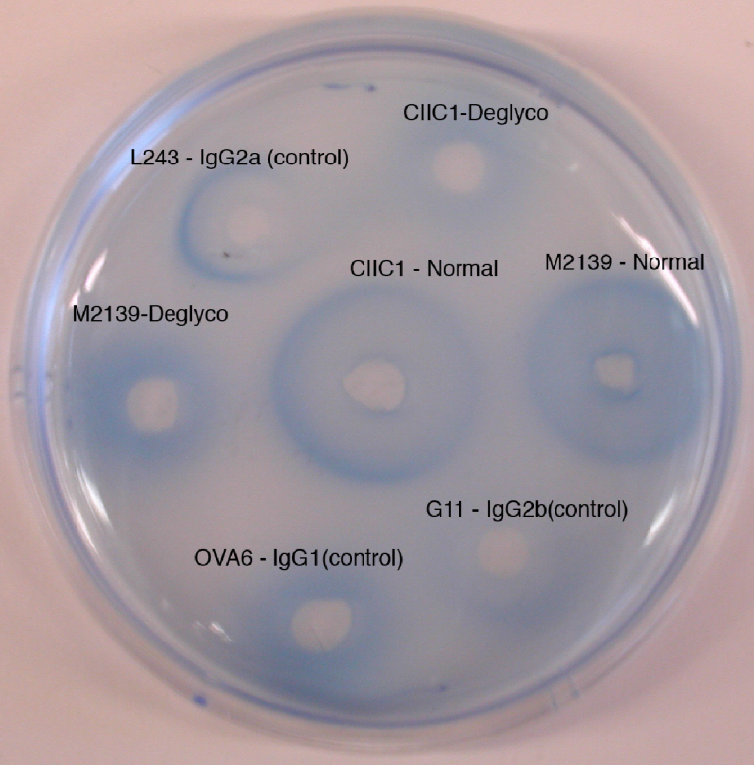
Antibodies bound to CII



Antibodies coated directly on plates







CIIC1-Deglyco

L243 - IgG2a (control)

CIIC1 - Normal

M2139 - Normal

M2139-Deglyco

G11 - IgG2b(control)

OVA6 - IgG1(control)