

## **A Novel heterophilic antibody interaction involves IgG4**

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**Short Title:** Human IgG4 binds to animal IgG.

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## **Abstract**

IgG4 has been implicated in a diverse set of complex pathologies - e.g. auto-immune pancreatitis (AIP), idiopathic membranous nephropathy - and carries unique features including lack of activation of the classical complement pathway and a dynamic Fab arm exchange. We recently showed that the rheumatoid factor (RF) like activity of IgG4 is achieved through a hitherto unknown, Fc-Fc (and not Fab-Fc as is the case in classical RF; CRF) interaction; hence the name, novel RF (NRF). Here we further explore the resemblance/difference between CRF and NRF. As heterophilic interactions of human IgM RF (CRF) are well known, we checked whether this is the case for IgG4. Human IgG4 showed variable reactivity to animal IgGs: reacting intensely with rabbit and mouse IgGs, but weakly with others. The binding to rabbit IgG was not through the Fab (as in CRF) but via the Fc piece, as was recently shown for human IgG (NRF). This binding correlates with the IgG4 concentration *per se* and could therefore be of diagnostic usage and incidentally explain some observed interferences in biological assays. In conclusion, here is defined a novel heterophilic antibody interaction, and is established the universality of the unique Fc-Fc binding, both involving IgG4.

## **Introduction**

A unique form of chronic pancreatitis displaying hypergammaglobulinemia, pancreatic lymphoplasmacytic inflammation and favorable response to corticosteroids has been recently dubbed “auto-immune pancreatitis” (AIP). [1, 2] Available genetic studies have further linked AIP susceptibility to the *HLA-DRB1\*0405-DQB1\*0401* haplotype, and with polymorphisms in Fc Receptor Like 3 (*FcRL3*) and *CTLA4* genes [3-6]. An outstanding finding in AIP is the existence of a high serum concentration for IgG4 which has been documented in 90% of patients [7]. This occurs in parallel to an abundant IgG4 positive plasma cell infiltration in the pancreatic tissue [8] collectively suggesting that IgG4 may have a decisive role in AIP pathogenesis. At present AIP is considered to be the pancreatic expression of a broader IgG4-associated systemic disease.[9]

The role of IgG4 in immune response and auto-immunity has not yet been fully elucidated, despite recent elegant studies implicating it in defined organ-specific auto-immunity e.g. idiopathic membranous nephropathy [10]. The biology of IgG4 is equally interesting. Indeed this Ig subclass is unique to the fact that it is unable to activate the classical pathway of complement and possesses a “dynamic lifestyle” given a continuous process of half-molecule exchange, referred to “Fab-arm exchange”[11,

12]. Moreover IgG4 can exist in sera of healthy subjects as anti-IgG antibody, alike the rheumatoid factor [13-15]. This is exacerbated in AIP patients where the rise in IgG4 concentration has helped us to understand the topology of IgG4 – IgG interaction. To our surprise this interaction was not through the classical Fab-Fc recognition in the rheumatoid factor (RF) (hence called classical RF; CRF) but via an unprecedented Fc-Fc binding allowing to dub this interaction as “novel rheumatoid factor” (NRF) versus CRF (the IgM rheumatoid factor) [16]. This has been since validated by at least another group [17]. Because the CRF is well known to react with the Fc piece of animal IgG [18] and this has been indeed part of its historical identity, at times used in diagnostics, we set to further assess if the human IgG4 may follow a similar interaction. If this was indeed the case the question shall arise as to if this is done through the same Fc-Fc interaction.

## **Results**

### **Human IgG4 binds to IgG from various animal species**

IgG4 remains a peculiar subclass of human IgG given several unique characteristics mentioned above. These include both its implication in unique pathophysiological situations as well as its inherent physicochemical properties. Paramount among the

latter are perhaps its dynamic Fab arm exchange [12] and the more recently identified rheumatoid factor activity which is executed through a unique Fc-Fc interaction unlike the classical Fab-Fc recognition of the IgM anti-IgG RF [16, 17]. Here we aim to take an incremental step in further characterizing human IgG4 RF (NRF) and its resemblance/dis-resemblance to CRF. One main characteristic of CRF is its reactivity to animal IgG [19]. This was used at times in diagnostic tests and has been a source of biological interference in clinical assays [20].

Animal IgGs showed a range of reactivity to human IgG4 purified from AIP patients. Mouse and rabbit IgGs had strong reactivity to human IgG4, guinea pig, bovine, goat and dog had intermediate reactivity, and finally sheep, horse and rat IgG scarcely reacted at all (Fig. 1). Because mouse IgG had such strong reactivity to human IgG4, we checked the reactivity of its different subclasses. There was intense reactivity of mouse IgG2a with human IgG4 but weak reactivity with the other 3 mouse IgG subclasses (Fig.2).

**Human IgG4 binds animal IgG via an Fc-Fc interaction.** Because rabbit (like mouse) IgG had strong reactivity to human IgG4, among the various animal IgGs tested, we assessed the topology of rabbit IgG reactivity to human IgG4 by Western blot and

ELISA. The identity of each IgG4, IgG4 F(ab')<sub>2</sub> and IgG4 Fc sample was confirmed by the reactivity with the corresponding HRP-labeled antibody: HRP-labeled anti-IgG4 Fc antibody reacted to purified IgG4 and IgG4 Fc, but not to IgG4 F(ab')<sub>2</sub>; HRP-labeled anti-human  $\kappa$  antibody reacted to IgG4 F(ab')<sub>2</sub>, but not to IgG4 Fc. We further checked whether the IgG4 Fc bound to rabbit IgG or not. A preparation of rabbit IgG was resolved on a 10 % SDS-PAGE under reducing conditions and then transferred onto a PVDF membrane. HRP-labeled anti-IgG4 Fc antibody and HRP-labeled anti-human  $\kappa$  antibody had no reactivity to rabbit IgG (Fig.3, lanes 1 and 2). HRP-labeled anti-IgG4 Fc antibody showed strong reactivity to rabbit IgG after lanes were incubated with purified IgG4 (Fig. 3, lane 3) or IgG4 Fc (Fig. 3, lane 5). However, HRP-labeled anti-human  $\kappa$  antibody showed no reactivity to rabbit IgG after the lane was incubated with IgG4 F(ab')<sub>2</sub> (Fig. 3, lane 4). These results indicated that it was IgG4 Fc rather than the Fab piece that bound to rabbit IgG, as for human IgG, and that therefore IgG4 binding to rabbit IgG is not due to antibody (Fab) activity.

### ***In vivo* assessment**

The binding of serum IgG4 to rabbit IgG was negligible in 130 healthy controls, as well as in patients with other autoimmune diseases including autoimmune hepatitis,

primary biliary cirrhosis, primary sclerosing cholangitis, systemic lupus erythematosus and Sjögren's syndrome, as well as those affected with other pancreatic diseases, i.e. chronic pancreatitis and pancreatic cancer. This was in contrast to the properties of serum IgG4 in a cohort of AIP patients in whom there were significantly elevated concentration of IgG4 that bound to rabbit IgG (Fig. 4).

In patients with AIP, serum levels of IgG4 bound to rabbit IgG were correlated well with the serum IgG4 level itself (correlation coefficient 0.899,  $p < 0.0001$ ), but not with serum rheumatoid factor levels (correlation coefficient -0.0723,  $p = 0.580$ ). For these patients, high serum IgG4 concentrations ( $>135\text{mg/dL}$ ) were found in 50 of 65 (76.9%) and the median IgG4 value was 711 mg/dL.

## **Discussions**

The present study demonstrated that human IgG4 obtained from AIP patients bound to a variety of animal IgGs, albeit with different affinities. High affinity was found for mouse and rabbit IgG, whereas weak or minimal affinity was found for sheep, horse and rat IgG. It is well established for human IgM rheumatoid factor (CRF) to have high affinity for rabbit IgG; indeed this is used in clinical testing for detection of human RF in rheumatoid arthritis for instance [19]. Accordingly, the reactivity of human IgG4

for animal IgG resembles that of RF in regard to the affinity of reactivity for rabbit IgG. We have meagre data to explain the difference in affinity of human IgG4 for various animal IgGs. In our study, human IgG4 reacted intensely to the mouse IgG2a subclass, but minimally to the other 3 mouse IgG subclasses, indicating that human IgG4 has different affinities for each subclass of an individual animal IgG. The low affinity of human IgG4 for some animal IgGs may be due to a low-affinity IgG subclass being purified from the whole IgG of animal by protein A or G affinity chromatography and available commercially. Alternatively, evolutionary changes in IgG structure may account for the difference. We need further experiments to role out the mechanism behind the strong variation in IgG4 binding to IgG from different species.

There are several interesting features for human IgG4 [11]: inability to activate the classical complement pathway [21], Fab arm exchange [12] and rheumatoid factor like activity due to Fc-Fc interaction [16, 17]. Hence we checked whether IgG4 binding for animal IgG was in fact conferred by its Fc piece, using rabbit IgG. Western blot analysis confirmed that IgG4 binding to rabbit IgG was conferred by the Fc piece of IgG4, as pertains for human IgG [16], indicating that this IgG4 reactivity cannot be regarded due to antibody activity or to the “classical” rheumatoid factor activity. In addition, ELISA showed that in our study each patient with autoimmune pancreatitis with a high serum

IgG4 concentration had a high serum level of IgG4 that bound to rabbit IgG, and the serum level of IgG4 bound to rabbit IgG closely correlated with the actual serum IgG4 concentration but not with serum rheumatoid factor levels. Hence, human IgG4 showed intense reactivity to animal IgGs, with an Fc-Fc interaction similar to that seen with human IgG subclasses. Furthermore, serum rheumatoid factor seems to have little effect to this interaction.

The present assay system using rabbit IgG should provides several promising utilities. Incidentally, it will provide an alternative assay system for measuring serum IgG4 levels at low cost, because it needs no IgG4 capture antibody. Second, it can combine with a RF assay system: thus because RF also binds to rabbit IgG, both IgG4 and RF activities can be measured together, when the respective HRP-labeled anti-IgG4 and anti-IgG or anti-IgM are used as tracer antibodies in a single assay system. However, in contrast, a high affinity of IgG4 for rabbit or mouse IgG may interfere in various assay systems in which rabbit or mouse antibody are used for capture, when samples with high serum IgG4 concentration are assayed.

Given that IgG4 binding to rabbit IgG mimics the (classical) RF, the role of IgG4 may mimic that of RF. In general and to date, it remains to be determined whether RF activity is beneficial or detrimental [22] i.e. Fc-Fc interactions, like low-affinity

polyreactive IgM rheumatoid factors may aid in the clearance of immune complexes by forming larger ones that are more effectively cleared [23] but conversely, and like high affinity IgG and IgA rheumatoid factors, they may have harmful effects such as deposition in blood vessels causing vasculitis or nephritis [24, 25].

In conclusion, the distinctly elevated serum IgG4 found in autoimmune pancreatitis can bind various animal IgGs, as does classical rheumatoid factor. This unique, novel and at presently universal, heterophilic interaction - IgG4-Fc binding to the Fc piece of animal IgG – may have several promising utilities and/or cause/explain interference in some biological assays but more generally helps further define the NRF vs. CRF classification.

## **Subjects and methods**

### **Subjects**

Serum samples were obtained from 65 AIP patients - 54 men and 11 women, aged 38-79 years (median age 62.4 years) - 111 patients with alcoholic or idiopathic chronic pancreatitis, 96 with pancreatic cancer, 40 with autoimmune hepatitis (AIH), 39 with primary biliary cirrhosis (PBC), 20 with primary sclerosing cholangitis (PSC), 13 with

systemic lupus erythematosus (SLE) and 7 with Sjögren's syndrome. 130 normal subjects were also included in the study. Serum samples were stored at -20 °C before use.

All 65 AIP patients fulfilled the revised diagnostic criteria proposed by Japan's Pancreas Society.[26] These include the following biological and radiological findings: elevated serum immunoglobulin including IgG4 and/or positive auto-antibodies e.g. anti-nuclear antibody and RF, the irregular narrowing of the main pancreatic duct - as evidenced by endoscopic retrograde cholangio-pancreatography (ERCP) - as well as an enlarged pancreas as assessed by ultra-sonography, computed tomography, or magnetic resonance imaging. Histological confirmation of lymphoplasmacytic infiltration and fibrosis in the pancreas was obtained for 13 of these patients.

All 111 patients with alcoholic or idiopathic chronic pancreatitis had either marked irregular dilatation of the main pancreatic duct or calcification of the pancreas. The diagnosis of pancreatic cancer was confirmed by histological findings in 38 patients and by both typical findings on imaging procedures and the clinical course in 58 patients.

All subjects provided written informed consent for invasive tests such as ERCP and liver biopsy. Blood samples were obtained after permission was received from the

patients. The institutional ethics committee approved the study.

### **Preparation of IgG4 from pooled sera of patients with autoimmune pancreatitis**

IgG4 was purified from pooled sera of patients with AIP by affinity chromatography. The IgG4 F(ab')<sub>2</sub> fraction and IgG4 Fc fractions were derived by digesting purified IgG4 with pepsin and papain, respectively[16]. Horseradish peroxidase (HRP) conjugation of purified IgG4 was performed using peroxidase labeling kit-NH<sub>2</sub> (DMT LK11, Dojindo Molecular Technologies, Inc. Maryland) according to the manufacturer's instructions. The peroxidase-conjugated mouse anti-IgG4 monoclonal antibody reacted to the Fc portion of IgG4 (South Biotech 9200-05). Goat anti-human  $\kappa$  light chain (Bethyl Laboratories, Inc, A80115P) was used to detect IgG4 F(ab')<sub>2</sub>.

### **Human IgG4 binding for Animal IgGs**

Micro ELISA plates (Nunc immunoplate 446612, Rochester, NY) were coated with various animal IgGs with concentrations from 0.15 to 10  $\mu$ g/ml. Animal IgGs used to assess the binding capacity of human IgG4 were the followings: pig (ICT F16031, Inter-Cell Technologies, Inc., Florida), chicken (ICT F18031), hamster (SC2713, Santa Cruz Biotechnology, Inc. California), guinea pig (SC 2711), goat (SC 2028), horse

(SC2714), sheep (SC2717), bovine (SC2709), dog (SC2710), rat (SC2026), rabbit (SC3888) and finally mouse (SC3877). We also checked the reactivity of human IgG4 to mouse IgG subclasses, i.e. IgG1 (ANC 278010, Ancell Inc. Bayport, USA), IgG2a (ANC 281010), IgG2b (ANC 284010) and IgG3 (ANC 287010). After overnight incubation at + 4°C the animal IgG solution was discarded and plates were blocked with 1 percent bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 10 mmole of ethylene diamine tetraacetic acid (EDTA). After three washes with pH 6.8 PBS containing 0.01% tween 20, immobilized animal IgG was then incubated with peroxidase-conjugated purified IgG4 in enzyme conjugate stabilizer solution (Stab-ELISA-rHRP Diluent/Stabilizer, Cygnus Technologies I-035, Southport, NC) (1:2000 dilution). The enzyme bound to the wells was incubated in the dark with tetramethylbenzidine substrate solution (TMB One-component Microwell Peroxidase Substrate, Kirkegaard & Perry Laboratories 53-00-01, Gaithersburg, ML). The reaction was stopped by adding stop solution (TMB One-Component Stop Solution Kirkegaard & Perry Laboratories 50-85-05). The optical density was determined at 450nm using an ELISA reader (Bio Rad Model550 Microplate Reader).

## **Western blot**

For Western blots (WB), rabbit IgG was resolved on 10 % sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions, with transfer onto polyvinylidene difluoride (PVDF) membrane (Bio Rad 163-0181, Hercules, CA). After blocking with 5% non-fat milk, the blot was incubated overnight with IgG4, IgG4 Fc and IgG4 F(ab')<sub>2</sub> from AIP patients. Lanes with IgG4 and IgG4 Fc were incubated with peroxidase-conjugated anti-human IgG4 Fc antibody (1:1000 dilution). The lane with IgG4 F(ab')<sub>2</sub> was incubated with peroxidase-conjugated anti-human  $\kappa$  antibody (1:1000 dilution). The blot was developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). To exclude non-specific binding of peroxidase-conjugated antibodies to rabbit IgG, blocked membrane was incubated directly with peroxidase-conjugated anti-human IgG4 Fc antibody and anti-human  $\kappa$  antibody, respectively.

## **Serum assays for IgG4 bound to rabbit IgG**

Micro ELISA plates (Nunc immunoplate 446612, Rochester, NY) were coated with rabbit IgG (3 $\mu$ g/well). After overnight incubation at +4°C the Ig solution was discarded and plates were blocked with 1 percent BSA in PBS containing 10 millimole of EDTA.

After the blocking solution was discarded, 1:5000-diluted serum samples were added. After three washes in pH 6.8 PBS containing 0.01 percent tween 20, immobilized complexes were then incubated with peroxidase-conjugated anti-IgG4 monoclonal antibody in enzyme conjugate stabilizer solution (Stab-ELISA-rHRP Diluent/Stabilizer, Cygnus Technologies I-035, Southport, NC) (1:2000 dilution). The enzyme bound to the wells was incubated in the dark with tetramethylbenzidine substrate solution (TMB One-component Microwell Peroxidase Substrate, Kirkegaard & Perry Laboratories 53-00-01, Gaithersburg, MD). The reaction was stopped by adding stop solution (TMB One-Component Stop Solution Kirkegaard & Perry Laboratories 50-85-05). The optical density was determined at 450nm using ELISA reader (Bio Rad Model550 Microplate Reader).

To construct the standard curve, pooled sera of AIP patients were serially diluted from 39 to 10,000 ng/ml of IgG4 and the optical density for each IgG4 value was plotted. The optical density of bound IgG4 concentration in each serum sample was converted to an absolute IgG4 value using this standard curve. The concentrations of standards were expressed as IgG4 values from the pooled patient sera. The linear correlation was obtained for each assay system between the absorbance of IgG4 bound to rabbit IgG and the IgG4 value from the pooled sera. The intra-assay variation was

less than 5.0%, and the inter-assay variation less than 7.5%. The HRP-conjugated second antibody reacted minimally to coated myeloma proteins.

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**Conflict of interest :** None

## Figure legends

**Figure 1.** ELISA showing human IgG4 reactivity for various animal IgGs. Various animal IgG was coated onto a microplate and then reacted with HRP-human IgG4. Human IgG4 shows a wide variety of reactivity to animal IgGs.

**Figure 2.** ELISA showing human IgG4 reactivity for mouse IgG subclasses. Each mouse IgG subclass was coated onto a microplate and then reacted with HRP-human IgG4. Human IgG4 shows strong reactivity to mouse IgG2a.

**Figure 3. The topology of IgG4–rabbit IgG interaction.** Western blotting was used to establish whether the Fc or the Fab portion(s) of IgG4 reacted to rabbit IgG. Rabbit IgG was blotted on each lane. HRP labeled anti-human IgG4 Fc or HRP-labeled anti-human  $\kappa$  light-chain showed no reactivity to rabbit IgG (lanes 1 and 2). HRP labeled anti-IgG4 Fc antibody reacted in lanes 3 and 5, which were previously incubated with purified IgG4 and IgG4 Fc, respectively. HRP labeled anti-human  $\kappa$  light-chain had no reactivity to lane 4, which was incubated with IgG4 F(ab')<sub>2</sub> (lane 4). These results indicated that IgG4 binds to rabbit IgG by its own Fc, and not by Fab as a classical RF. Experiments

were repeated three times with identical results.

**Figure 4. Scattergram of serum levels of IgG4 bound to rabbit IgG.** Serum concentrations of IgG4 that bound to rabbit IgG were significantly elevated in patients with autoimmune pancreatitis, whereas those in other conditions were remained in lower levels.

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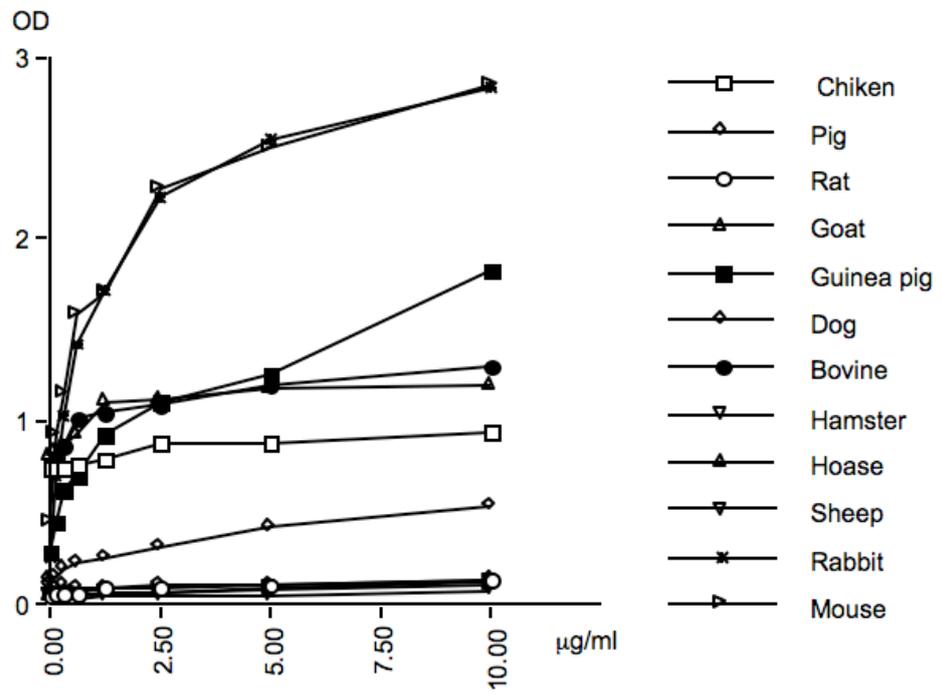


Figure 1

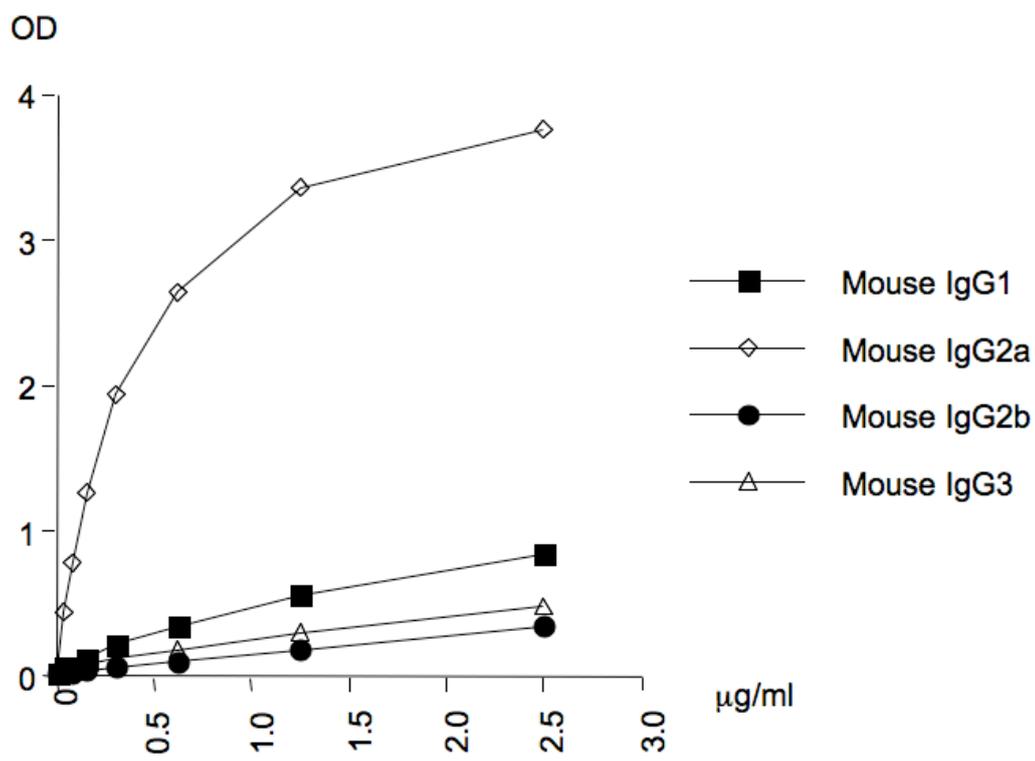


Figure 2

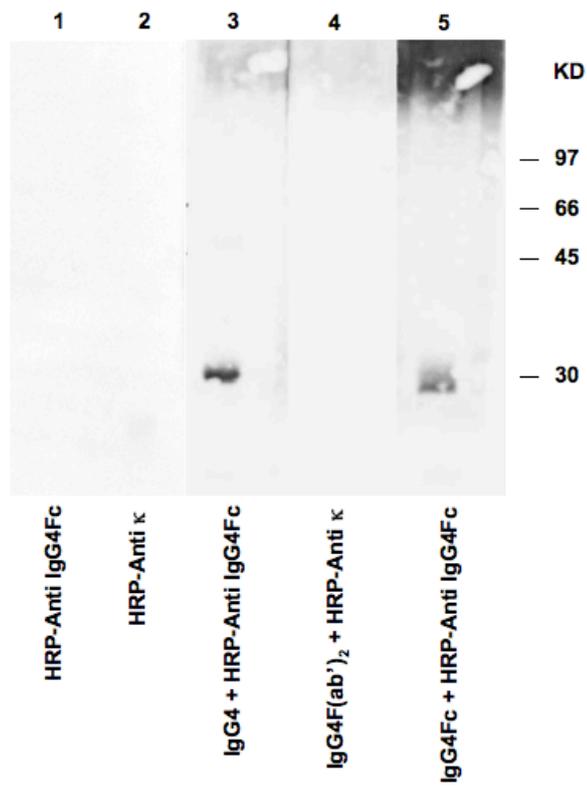


Figure 3

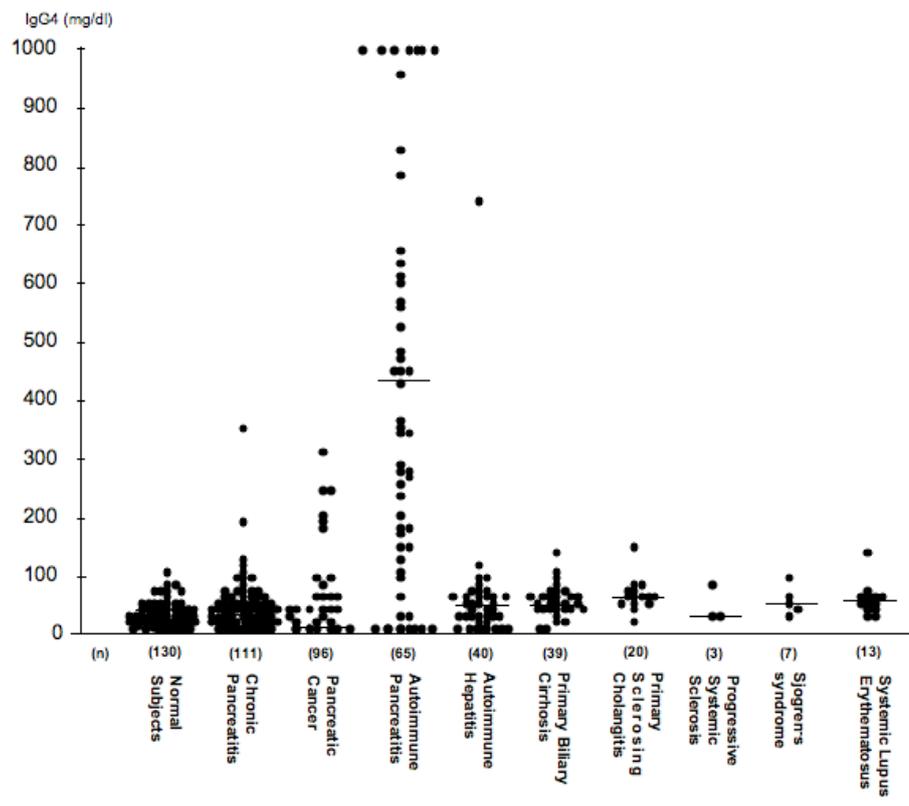


Figure 4