ELUCIDATING THE ROLE OF SERUM IgA

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Abstract

Natural antibodies, which are constitutively produced by B-lymphocytes in a healthy individual, exhibit poly-reactivity and non-specificity to antigens. Recently, it was shown that natural IgG (nIgG) cooperates with glycan-binding proteins (lectins) such as H-ficolin during infection-inflammation conditions to facilitate effective clearance of pathogens by phagocytes. However, the loss of control over these protein interaction systems often leads to immune complex disorders such as Systemic Lupus Erythematosus (SLE). While the functions of other natural antibodies (nIgM, nIgG) had been better characterised, the role of serum natural IgA remain relatively unexplored. Here, we characterised the interaction between serum IgA and other serum proteins under infection-inflammation or normal conditions. Co-immunoprecipitation studies revealed that serum IgA exhibits low propensity to form immune complexes under inflammatory conditions. Surface Plasmon Resonance analysis showed that serum IgA binds H-ficolin, but with kinetics opposite to that of nIgG. Deglycosylation of serum IgA reduced its interaction with H-ficolin. Furthermore, we showed that serum IgA could possibly regulate serum IgG function and immune complex formation. Further research may focus on therapeutically skewing natural antibody production towards IgA and usage of glyco-IgA peptides mimics to intervene in immune complex diseases.

(189 words)
**List of abbreviations**

Co-IP: Co-immunoprecipitation

Fc region (of antibody): Fragment crystallisable

IgA deglycosylated using the enzyme PNGaseF: DIA-P

IgA deglycosylated using the enzyme Galactosidase: DIA-G

MBL: Mannose Binding Lectin

Natural IgG: nIgG

Natural IgM: nIgM

SPR: Surface Plasmon Resonance

SLE: Systemic Lupus Erythematosus
**Introduction**

The role of natural antibodies, particularly nIgG and nIgA, in immunity had remained enigmatic over five decades since its discovery. Natural antibodies are constitutively produced throughout the human lifespan but are poly-reactive and do not have high specificity for their targets (Boyden, 1966; Michael, 1969). This is in contrast to their better characterised antigen specific counterparts, which home in to bind specific epitopes on the pathogen surface to exert their effector function (Muramatsu et al., 1999). As such, natural antibodies have been hitherto viewed as “useless” - lacking an effector function in immunity (Vollmers and Brandlein, 2006).

The past ten years or so has seen a revival of interest in research on natural antibodies. The role of natural IgM (nIgM) has been shown to be important for innate front-line neutralisation of pathogens or binding to certain self-antigens for clearance of apoptotic cells (Vollmers and Brändlein, 2006). Recently, a better understanding was gained on the role of natural IgG (nIgG). nIgG collaborates with lectins like ficolin and mannose binding lectin (MBL) to recognize pathogens, enhancing effectiveness of pathogenic clearance (Panda et al., 2013). Infection-inflammation condition (lowered pH) further increases the affinity of the nIgG:lectin complex for pathogens and improves bacterial clearance by innate immune cells such as macrophages. Furthermore, the generation of pro-inflammatory cytokines (IL8, TNF, IL6) by these macrophages are greatly increased. However, effective as it is, sustained activation of such similar systems, coupled with poor clearance, are known to lead to chronic inflammatory conditions and widespread immune complex formation in autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (Ohl and Tenbrock, 2011).
SLE is a chronic autoimmune disease in which auto-antibodies produced are directed against self-antigens (nuclear components), causing widespread immune complex deposition in various tissues in the body. In addition, SLE patients exhibit poor clearance of such immune complexes due to deficient serum complement components, such as the C1 complex, C2 and C4 (Boes et al., 2000). These immune complexes induce widespread cytokine release, causing a chronic inflammatory condition (Mathsson et al., 2007). Such a condition further facilitates immune complex formation, setting up a vicious cycle that drives continuation of the disease state. The cause of SLE is multifactorial, involving genetics, environments and hormones, and it is hard to pin down on a single factor as the major cause (Munoz et al., 2005). Regardless of the cause of the disease, certain homeostatic factors would have been lost to allow the disease to progress. This motivated us to study the immune complexes formed – to examine if the H-ficolin and IgG are the molecular drivers of immune complex formation, and to tease out which regulatory homeostatic factors are missing.

Humans have two isotypes of IgA, the monomeric serum IgA and dimeric mucosal secretory IgA. The role of secretory IgA has been relatively well studied. Secretory IgA prevents pathogenic and commensal bacterial invasion across the mucosal epithelial layer, preventing systemic infection while simultaneously maintaining a physiologically necessary symbiotic relationship with commensal bacteria (Pabst, 2012). In contrast, the role of serum IgA, especially the natural form, remains relatively unexplored. Earlier studies have shown that IgA can induce both pro- and anti-inflammatory responses from innate immune cells (Bakema and van Egmond, 2011). Furthermore, serum IgA has been shown to interact with many other proteins and glycoproteins of which the functions and mechanisms are not fully characterised (Bakema and van Egmond, 2011). As such, another part of this study was aimed at
exploring the way serum IgA and other proteins, such as ficolins, interact in the serum and the outcome of such interactions (regulatory, homeostatic, anti-inflammatory, pro-inflammatory).

Our results from co-immunoprecipitation studies reveal that serum IgA exhibits low propensity to form immune complexes under inflammatory conditions. Using SPR, we discovered that serum IgA binds H-ficolin, but with kinetics opposite to that of nIgG (Panda et al., 2013). Serum IgA binds H-ficolin with stronger affinity under normal conditions but with weaker affinity under infection-inflammation conditions. Deglycosylated IgA showed loss of interaction with H-ficolin indicating the importance of the glycan chains for H-ficolin interaction. Furthermore, we showed that serum IgA could possibly regulate nIgG function and immune complex formation under normal conditions. Taken together, we showed that IgA could possibly be one of the missing or lowered homeostatic factors in SLE, given that SLE patients show lowered IgA levels.

Materials and Methods

Antibodies and Reagents
Wild-type IgA antibodies, EDEGLY Enzymatic Protein Deglycosylation kit, goat anti-human IgA primary antibodies and goat anti-human IgG primary antibodies were all purchased from Sigma. The purity and integrity of IgA were verified by the manufacturer to be derived from the serum of healthy donors. Goat anti-human H-ficolin primary antibody was purchased from R&D systems. Rabbit anti-human mannose binding lectin (MBL) antibodies were purchased from Abcam. Rabbit anti-goat horseradish peroxidase and goat anti-rabbit horseradish peroxidase were purchased from Dako. Anti-IgA beads and Protein G beads were purchased from Invitrogen and GE Healthcare Life Sciences, respectively. HEK293T (ATCC) cells were transfected
with pSecTag Mammalian Expression Vector (Life Technologies) containing the H-ficolin gene using lipofectamine 2000 (Invitrogen). Recombinant H-ficolin was then pulled-down and purified from cell culture supernatant using Nickel-NTA beads (Qiagen).

**pH Measurements**
Healthy donor and SLE serum samples were diluted 1:10 using 0.9% saline solution. pH measurements were then made using a Sartorius Docu-pH Meter.

**Deglycosylation of IgA**
270 µl of IgA from human serum containing 300 µg of IgA was incubated with 4 µl of PNGase F and Galactosidase (EDEGLY Enzymatic Protein Deglycosylation Kit) in a reaction buffer containing 250 mM sodium phosphate (pH 7.0) for five days at 37°C. Deglycosylated IgA was then pulled down using 30 µl of anti-IgA beads at 4 ºC overnight with rotation. Beads were packed into a spin column and washed with 1X PBS (pH 7.0). Deglycosylated IgA was then eluted from the beads using glycine elution buffer, pH 3.0, into tubes containing Tris buffer, pH 9.0 (1/10 volume of elution buffer).

**Purification of IgG from healthy human serum**
Protein G-Sepharose (GE Healthcare Life Sciences) was placed in to a column (Sartorius) and reconstituted with 20 mM sodium phosphate buffer, pH 7.0. Serum diluted in 1:1 ratio in the equilibration buffer was loaded into the column and incubated for 30 min at room temperature. Beads were then washed ten times with equilibration buffer to remove unbound proteins. IgG was eluted using 0.1 M glycine buffer, pH 3.0, into tubes containing Tris buffer, pH 9.0 (1/10 volume of elution buffer).
**In vitro simulation of normal and infection-inflammation conditions**

Infection-inflammation is associated with local mild acidosis of pH 6.5 (Martinez et al., 2006). The blood calcium levels drop from ~2.5 mM to 2.0 mM (TranVan Nhieu et al., 2004; Prince et al., 2006; Eichstaedt et al., 2009). We simulated normal, healthy physiological conditions using TBS buffer, pH 7.4 containing 25 mM Tris and 145 mM NaCl. For infection-inflammation conditions, we used MBS buffer, pH 6.5, containing 25 mM Tris and 145 mM NaCl. These buffers are commonly used by others to simulate the abovementioned conditions (Miyazawa and Inoue, 1990; Gu and Lee, 2006; Zhang et al., 2009; Panda et al., 2013; Panda et al., 2014).

**Surface Plasmon Resonance (SPR) real-time biointeraction analysis**

The BIAcore 2000 SPR instrument was used to show interaction between proteins. A CM5 chip was immobilized with GlcNAc-BSA (Dextra Labs, UK) according to the manufacturer’s specifications. For the experiments, H-ficolin in running buffer was first injected for binding to the immobilised GlcNac. To analyse the binding of IgA to H-ficolin, IgA in running buffer was injected in increasing concentrations over the immobilised H-ficolin. Bound proteins were removed with injection 15 µl of 0.1M NaOH in between cycles. Flow rate was maintained throughout at 30 µl/min. The running buffer for normal condition and infection-inflammation conditions was TBS and MBS buffer, respectively, as described on page 3. The BIA evaluation 3.2 software was used to calculate the binding affinity values (K_D). The plots shown are representative of three independent experiments.
Pull-down of immune complex and co-immunoprecipitation (co-IP)

Immune complex pull-down from healthy and SLE serum samples was performed by incubating 10 µl of Protein G beads (GE Healthcare Life Sciences) with serum for 2 h at 25°C with shaking. Beads were washed three times with 0.9% saline prior to incubation. For experiments using anti-IgA beads (Life Technologies), 5 µl of beads were first reconstituted with TBS or MBS buffer. IgA or deglycosylated IgA (D IgA) and H-ficolin were then added to the beads in 100 µl of buffer and incubated for 2 h with shaking. For both sets of experiments, beads were washed 4 times with TBST and MBST (TBS or MBS buffer with 0.1% Tween 20 (Sigma)) after incubation. Beads were then eluted using 50 µl of 1x SDS-PAGE Loading Buffer and analysed using 12% SDS-PAGE electrophoresis followed by Western blotting using specific antibodies.

Statistical Analysis

Error bars indicate mean ± s.e.m. Differences between means were analysed using the two-tailed Student’s-test. Significance was set at a P-value of 0.05. *P<0.05; **P<0.01.

Results and Discussion

IgA does not form immune complexes under chronic infection-inflammation conditions

SLE is an immune complex disease characterised by immune complex deposition in many sites of the body. Patients with SLE appear to suffer from chronic infection-inflammation condition and altered levels of serum proteins, such as ficolins, and elevated levels of IgG and IgM (Boes et al., 2000). IgG has been widely reported as the main player in such immune complexes (Kotzin, 1997; Boes et al., 2000). Taken together, these conditions are ideal to study the IgA ‘interactome’. Studying the immune
complexes formed would allow us to gain an insight on whether IgA is part of such immune complexes and its interaction with other serum proteins such as H-ficolin.

Measurements conducted on SLE serum confirmed that pH was indeed lower compared to normal serum (Figure 1a,b) It should be noted that pH values obtained for both healthy donors and SLE patients were abnormal and deviated from the normal blood physiological pH range of 7.35-7.45. This could have been due to factors such as temperature of measurement and storage conditions. However, measured values were consistently similar within the two groups: healthy or SLE patients. Importantly, this established that SLE patients indeed displayed lower serum pH values compared to that of healthy control serum.

Western blot showed IgG levels to be elevated in SLE serum while IgA levels appear to be lowered (Figure 1c). Results from immune complex pull-down using anti-IgG beads (protein G beads) from SLE patient serum showed complexes formed with IgG, Mannose Binding Lectin (MBL), and H-ficolin participating in the interactome formation. In normal human serum samples, only IgG and H-ficolin were co-immunoprecipitated (co-IP) but was substantially lower than that in SLE patients. Interestingly, IgA was not found to be part of such immune complexes.
Figure 1. Serum pH, protein levels and immune complex detected in healthy and SLE samples. (a) Serum pH in healthy and SLE patient samples. The labels indicate the experimental serum numbers. Five members per group were tested. ** P<0.01 (b) Table showing the serum pH and clinical serum numbers of healthy and SLE samples (c) Positive control western blot of whole serum from healthy and SLE patients to show presence of proteins. (d) Immune complex pull-down from serum using Protein G beads. Proteins bound to IgG were detected using western blot analysis. Two healthy and five SLE samples were tested for (c) & (d).
These results were confirmed using anti-IgA beads. IgA was found to bind H-ficolin in both normal healthy human serum and SLE serum. No other proteins were detected to complex with IgA-ficolin in both normal healthy and SLE serum (Figure 2). The down-regulation of serum IgA might have contributed to the exacerbation of SLE. Thus we hypothesized at this point that IgA may regulate IgG, which is the main driver of immune complex formation in SLE. The above results indicate that although IgA is present albeit in relatively lower amounts in SLE serum, it does not form immune complexes, or, at best forms unstable complexes under infection inflammation conditions.

![Figure 2. Immune complex co-IP from healthy and SLE patients human serum using anti-IgA beads. Only IgA-H-ficolin was found to interact. IgG and MBL was not co-immunoprecipitated. Two healthy and five SLE serum samples were tested.](image-url)
IgA shows weaker binding affinity for H-ficolin under infection-inflammation conditions

Since IgA does not form immune complexes under lowered pH conditions (chronic inflammation condition as mimicked for SLE), we reasoned that perhaps IgA displays lower affinity for H-ficolin under inflammatory conditions but would have stronger affinity under normal conditions. We thus performed co-IP using anti-IgA beads to compare IgA and H-ficolin interaction between normal and infection-inflammation conditions. As shown in figure 3, H-ficolin indeed showed weaker binding (very faint bands) under infection-inflammation conditions.

![Figure 3](image)

Figure 3. Co-IP of IgA-H-Ficolin using anti-IgA beads. 1µg of wild-type human serum IgA or deglycosylated IgA and H-ficolin (0.5 µg or 2 µg) was incubated with anti-IgA beads for 2 hours. Beads were then washed and eluted by boiling beads in 1X SDS PAGE sample buffer. Western blot analysis was then carried out.

IgA glycans are important for IgA-H-ficolin interaction

IgA is naturally glycosylated as part of the post-translational modification (Figure 5). Many reports in the past indicated that glycan chains on IgA appear important for IgA-pathogen or IgA-protein/glycoproteins interaction (Perrier et al.,
However, the precise mechanism of action involving the glycan moiety of IgA remained unclear. Since H-ficolin is a lectin, we decided to explore whether the glycans in IgA, particularly those localized to the CH2-CH3 domain (see Figure 5 and 6), would contribute to IgA:H-ficolin interaction. We thus deglycosylated IgA using the enzyme PNGaseF (DIgA-P) and used it for co-IP with H-ficolin using anti-IgA beads to pulldown potential immune complexes of IgA:H-ficolin (see Figure 3). (Appendix Figure A and B show deglycosylated IgA under different enzyme treatments and over different incubation times). PNGaseF removes N-linked sugars at the CH2-CH3 domain of IgA (Figure 5 and 6). As shown from the results in figure 3, DIgA-P lost interaction with H-ficolin (except for a single band at 0.5 µg H-ficolin). This suggests that perhaps the glycans were important for IgA and H-ficolin interaction. To gain better insight into the nature of IgA H-ficolin interaction, we next deglycosylated IgA using galactosidase (DIgA-G) and used it for the same co-IP experiment. Galactosidase cleaves off part of the glycan chains at both the O- and N-linked regions of IgA. Figure 4 shows that H-ficolin displayed weak binding to DIgA-G under normal conditions and virtually, no binding under infection-inflammation conditions. Consistently, DIgA-P shows a strong band at 2 µg H-ficolin under normal conditions.

Taken together, the results suggest that the glycan chains of IgA are: (1) important for IgA and H-ficolin interaction; (2) the length of the carbohydrate chains contributes to variable strength of binding; (3) Specific sugar residues are important for binding.
Figure 4. Co-IP of DlgA-P, DlgA-G and H-Ficolin using anti-IgA beads. 1µg of both types of deglycosylated serum IgA and H-ficolin (0.5 µg or 2 µg) was incubated with anti-IgA beads for 2 hours. Beads were then washed and eluted by boiling beads in 1X SDS PAGE sample buffer. Western blot analysis was then carried out.

Figure 5. Crystal structure of IgA and sites of glycosylation. Carbohydrate chains are shown as yellow chains. Regions of O-linked carbohydrate chains are located in the cyan box while regions of N-linked glycosylation are in the purple circle. Sites of carbohydrate chains cleavage by PNGase F and Galactosidase are shown by the red and dark blue lines, respectively.

Adapted from Mattu T S et al. J. Biol. Chem. 1998;273:2260-2272

Figure 4. Co-IP of DlgA-P, DlgA-G and H-Ficolin using anti-IgA beads. 1µg of both types of deglycosylated serum IgA and H-ficolin (0.5 µg or 2 µg) was incubated with anti-IgA beads for 2 hours. Beads were then washed and eluted by boiling beads in 1X SDS PAGE sample buffer. Western blot analysis was then carried out.

Adapted from Mattu T S et al. J. Biol. Chem. 1998;273:2260-2272
Aberrant glycosylation of IgA possibly leads to increased immune complex formation

It would have been myopic on our part if we did not consider other diseases caused by immune complex formation, such as IgA nephropathy and Henoch–Schönlein purpura. In these diseases, IgA is reportedly the major cause of immune complex formation. IgA nephropathy is caused by immune complexes involving IgA, depositing in the kidney glomeruli (Wyatt and Julian, 2013). Henoch–Schönlein purpura is caused by immune complexes involving IgA deposition in blood vessels (Roberts et al., 2007).

Further literature reviews show that patients suffering from such diseases have elevated levels of IgA and, importantly, aberrant glycosylation of IgA at the O-linked region. Apparently, such inappropriately glycosylated IgA causes conformational changes, leading to increased immune complex formation. In light of these findings, the bands that appeared in a seemingly random manner in the co-IP experiments could possibly be explained by the increased tendency of such ‘deviant’ antibodies for forming immune complexes. One might argue that this seems farfetched, but
henceforth, the mechanisms which govern immune complex formation (especially at localised niches in the body) in these diseases have yet to be fully explained. It is also interesting to note that, as is consistent with the IgA:H-ficolin co-IP result (see figures 3 and 5), the IgA:H-ficolin complexes which show up randomly do so only under normal conditions and not under infection-inflammation conditions. Therefore, this lends credence to our speculation that IgA:H-ficolin interaction is stronger under normal conditions.

Natural IgA binds H-ficolin but with kinetics different from that of natural IgG

Having established the behaviour of IgA under both conditions, we decided to quantify the binding affinity of IgA to H-ficolin through SPR analysis. Doing so would allow us to have a firmer grasp of how IgA interacts with H-ficolin under both conditions. It would also allow us to compare and contrast the kinetics of other established binding partners in the serum such as natural IgG and H-ficolin.

Panda et al. (2013) had shown that natural IgG binds weakly to H-ficolin under normal conditions but it binds strongly under infection-inflammation conditions, with a 589-fold increase in affinity. In this study, we revealed that IgA binds more strongly to H-ficolin under normal conditions but weaker under infection-inflammation conditions (confirming earlier Western blot results). In this case, the increase in affinity is rather subtle; at only about 2 fold lower (Figure 7).

Comparison between IgA and IgG under normal or infection–inflammation conditions showed that IgA had much higher affinity for H-ficolin under normal conditions (13.6-fold higher affinity versus IgG) but showed drastically weaker affinity under infection-inflammation conditions (73.6-fold weaker affinity versus IgG). Figure 8 shows the SPR curves of wild-type IgA and H-ficolin interaction.
### Normal Conditions (TBS, pH 7.4)

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### Infection-Inflammation Conditions (MBS, pH 6.5)

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**Figure 7.** SPR analysis of the binding kinetics of IgA to H-ficolin. Tables show KA and KD values of monomeric serum IgA binding to H-ficolin under Normal and Infection-Inflammation simulated conditions. 3 independent runs were done for each condition using TBS and MBS buffers. All data was fitted using a 1:1 Langmuir Binding Model using BIAEvaluation software (General Electric). KD values for nIgG under both conditions are shown at the right of the tables for comparison. Fold difference comparisons are in the text on the right.

**IgA: H-ficolin affinity:**
Stronger under normal vs infection-inflammation
Fold difference = 7.29×10⁸ / 4.29×10⁻⁸ = 1.69

**IgG: H-ficolin affinity:**
Stronger under infection-inflammation vs normal
Fold difference = 5.84×10⁻⁸ / 0.99×10⁻⁸ = 589

**IgA and IgG affinity to H-ficolin comparison:**
Normal: IgA stronger
Fold difference = 5.84×10⁻⁸ / 4.29×10⁻⁸ = 13.6

Infection-Inflammation: IgG stronger
Fold difference = 7.29×10⁸ / 0.99×10⁻⁸ = 73.6

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**Figure 8.** SPR analysis of the binding affinity between wild-type serum IgA and H-ficolin under normal conditions (representative figure). GlcNAc was immobilized on CM5 chip followed by injection of H-ficolin for 300 s (association time) and buffer for 300 s (dissociation time). Increasing concentrations of IgA were then injected over the bound H-ficolin under similar run conditions as indicated. Fitted curves are shown in red.
Real-time biointeraction analysis confirms IgA glycans are important for IgA:H-ficolin interaction

SPR analysis showed that serum IgA deglycosylated by PNGase F (D IgA-P) completely abrogated binding to H-ficolin under both normal or infection-inflammation conditions (Figure 9). In contrast, D IgA-G showed slight binding under normal conditions but not under infection-inflammation conditions (Figure 10). Thus, these SPR results confirmed our previous co-IP results using D IgA-P and D IgA-G. At this point, it is worth noting that the SPR experiments are based on a different principle to that of the co-IP experiments conducted previously (SPR is based on H-ficolin bound to a solid surface versus the fluid phase nature of the co-IP experiments). Therefore, one would not be able to discern if random immune complexes have formed from analysing the SPR curves. Also, one could possibly expect different results from the co-IP experiments.

Figure 9. SPR analysis of the binding affinity between D IgA-P and H-ficolin under normal conditions (representative figure). GlcNAc was immobilized on CM5 chip followed by injection of H-ficolin for 300 s (association time) and buffer for 300 s (dissociation time). Increasing concentrations of D IgA-P were then injected over the bound H-ficolin under similar run conditions as indicated. Curves were not fit since the software was unable to do so and the curves indicate no binding occurred. The dissociation curves starting on the same level as the baseline curve indicates no binding occurred.
IgA possibly downregulates IgG immune complex formation under normal conditions but allows for increased complex formation under infection-inflammation conditions.

Given the contrasting binding kinetics of IgA when compared to IgG, and taking into consideration the results of our previous experiments, we explored the possibility of IgA regulating IgG immune complex formation, specifically with H-ficolin.

A ‘competitive’ co-IP was set up. IgA, IgG and H-ficolin were incubated with anti-IgA beads. If IgA did indeed bind H-ficolin with higher affinity under normal conditions, more H-ficolin would be co-immunoprecipitated with IgA, and less H-ficolin in complex with IgG would be washed away. Under infection-inflammation conditions, given the 589-fold increase in affinity of IgG for H-ficolin, we would expect the opposite scenario (a drastic decrease in H-ficolin bound to IgA).

Indeed, results from the experiment showed that this appeared to be the case (Figures 11). Together with the earlier SPR analysis data, we hence surmise that IgA
possibly plays a regulatory role under normal physiological conditions, maintaining low levels of immune complex formation and preventing immune over-activation (e.g. release of pro-inflammatory cytokines by macrophages). In consideration of greater affinity of IgA for H-ficolin under normal condition, the native glycosylated IgA plausibly competes against IgG for H-ficolin to pre-empt IgG:ficolin immune complex formation and thereby maintains homeostasis. Under infection-inflammation conditions, IgA relinquishes its binding to H-ficolin, and IgG’s greatly increased affinity for H-ficolin would result in the formation of IgG:ficolin complexes which facilitates effective opsonisation and phagocytosis of pathogens. A model for serum IgA’s regulatory role and a workflow schematic of the experiments performed are shown in figures 12 and 13, respectively.

Figure 11. Western Blot for ‘Competitive’ co-IP of IgA versus IgG for H-ficolin binding. 2µg of IgA, 1 µg of H-ficolin and increasing amounts of IgG (0, 0.5, 1, 2 µg) were incubated with IgA beads for 2 hours. Beads were then washed and eluted by boiling beads in 1X SDS PAGE sample buffer. Western blot analysis was then carried out.
Future work and Perspective

IgG has been shown to be aberrantly glycosylated in SLE patients (Tomana et al., 1992). However, thus far, no studies have been done on the glycosylation status of IgA in SLE patients. Therefore it would be interesting to study the glycosylation status of the antibodies in the serum of those individuals used for the present study, in particular IgA. Validating that serum IgA in these patients is ‘properly’ glycosylated would point out that IgA’s low propensity to form immune complexes in SLE patients is due to (1) lowered pH due to chronic inflammatory conditions and (2) appropriate glycosylation patterns. In such patients, it could be possible that only IgG shows inappropriate glycosylation, adding to the severity of the disease. In contrast, given the differences in antibody isotype trafficking, IgA could still be properly glycosylated. As mentioned before, in the diseases IgA Nephropathy and Henoch–Schönlein purpura, inappropriate glycosylation of IgA are the causes of IgA immune complex formation. Taken together, all sources of evidence indicate that inappropriate glycosylation of IgA is sufficient for immune complex formation (involving IgA). We should also note that elevated levels of IgA do not drive IgA immune complex formation (Mestecky and Tomana, 1997; Mestecky et al., 2002).

Our studies have highlighted that glycans decorating IgA appear to be important for interaction with H-ficolin. However, the exact mechanism of such an interaction has not been characterised. The mechanism of interaction between natural IgG and H-ficolin at the molecular level, under ‘normal’ and ‘infection-inflammation’ was recently characterised using hydrogen deuterium exchange mass spectrometry (HDMS) (Panda et al., 2014). The interaction of IgA with H-ficolin could perhaps be revealed using similar techniques.
So far, all our experiments have been *in vitro*, as a proof-of-concept. Future studies should include cell-based assays and/or *in vivo* experiments using mice, to validate the observed effector functions of IgA *in vitro*.

Immune complex diseases are some of the most common immune complex diseases. Thus far, no cure has been developed for these diseases and treatment is mainly aimed at alleviating the symptoms that cause suffering. Common treatments include glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive drugs (Tsokos, 2011). Our studies revealed the possibility of developing IgA Fc region glycol-peptide mimics that works to antagonise IgG immune complex formation, thereby exploiting the benefits brought about by peptide drugs (Craik et al., 2013). Our results also indicate that therapeutically skewing natural antibody production (using cytokines, as shown in past research) towards serum IgA production could possibly be another treatment option for individuals afflicted with immune complex diseases (Kim et al., 2007).
Figure 12. Proposed model of IgA, IgG, H-ficolin interaction. Under normal conditions, IgA sequesters H-ficolin and lowers availability of it for complexing with IgG, regulating immune complex formation of IgG and preventing immune overactivation (e.g. release of pro-inflammatory cytokines by phagocytic cells). Under infection-inflammation conditions, IgA relinquishes its binding to H-ficolin, and IgG’s greatly increased affinity for H-ficolin facilitates effective opsonisation and phagocytosis.

Primary Observation:

Hypothesis:

Glycans important for binding?

Finding:

Yes

Yes

Discussion:

- Normal Condition: Regulation of immune complex formation & prevention of immune over activation
- Infection-inflammation: Effective Opsonisation & Phagocytosis through FcYR Receptor
- Importance of skewing B-cell antibody production to IgA in immune complex diseases (e.g. SLE)?
- IgA Fc Glycopeptide mimic drug for immune complex diseases?
- Future work: Cell-based in-vitro and in-vivo work, SLE IgA glycosylation studies, IgA:H-ficolin interaction mechanism

Figure 13. Workflow model of the project. Please see text for more details.
References


Pabst, O. (2012). New concepts in the generation and functions of IgA. Nature reviews Immunology 12, 821-832.


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Appendix

Figure A. Native PAGE followed by western blot analysis of wild-type serum IgA deglycosylated with indicated enzymes for time periods of 1 or 4 days. P+O; PNGaseF and O-glycosidase incubation.
Figure B. SDS PAGE followed by western blot analysis of wild-type serum IgA deglycosylated with indicated enzymes for time periods of 1 to 4 days. NC; negative control.
Figure C. Native PAGE followed by silver staining of D lgA-G and D lgA-P. All lanes show only a single band assuring that, at least qualitatively, the D lgA retains its high molecular weight tertiary structure.