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The constant region affects antigen binding of antibodies to DNA by altering secondary structure

Yumin Xia^{a,b}, Alena Janda^b, Ertan Eryilmaz^c, Arturo Casadevall^b, and Chaim Putterman^{a,b}

^aDepartment of Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

^bDivision of Rheumatology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

^cDepartment of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Abstract

We previously demonstrated an important role of the constant region in the pathogenicity of anti-DNA antibodies. To determine the mechanisms by which the constant region affects autoantibody binding, a panel of isotype-switch variants (IgG1, IgG2a, IgG2b) was generated from the murine PL9-11 IgG3 autoantibody. The affinity of the PL9-11 antibody panel for histone was measured by surface plasmon resonance (SPR). Tryptophan fluorescence was used to determine wavelength shifts of the antibody panel upon binding to DNA and histone. Finally, circular dichroism spectroscopy was used to measure changes in secondary structure. SPR analysis revealed significant differences in histone binding affinity between members of the PL9-11 panel. The wavelength shifts of tryptophan fluorescence emission were found to be dependent on the antibody isotype, while circular dichroism analysis determined that changes in antibody secondary structure content differed between isotypes upon antigen binding. Thus, the antigen binding affinity is dependent on the particular constant region expressed. Moreover, the effects of antibody binding to antigen were also constant region dependent. Alteration of secondary structures influenced by constant regions may explain differences in fine specificity of anti-DNA antibodies between antibodies with similar variable regions, as well as cross-reactivity of anti-DNA antibodies with non-DNA antigens.

Keywords

Systemic lupus erythematosus; anti-DNA antibodies; Antigen-antibody interactions

1. Introduction

Antibodies (Abs) to DNA play a key role in the pathogenesis of systemic lupus erythematosus (SLE), in particular lupus nephritis. Abs to DNA are believed to contribute to renal injury in SLE patients by cross reactivity with glomerular antigens (Yung and Chan,

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Address Correspondence to: Chaim Putterman, MD, Division of Rheumatology, Albert Einstein College of Medicine, F701N, 1300 Morris Park Ave., Bronx, NY 10461, Tel. 718-430-4266, Fax: 718-430-8789, chaim.putterman@einstein.yu.edu.

Conflict of Interest

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2008). Other mechanisms of pathogenicity include renal damage by penetration into living cells with modulation of gene expression and cell metabolism (Jang et al., 2009; Qing et al., 2008), enhancement of kidney cell proliferation (Yung et al., 2005), and induction of phenotypic changes in mesangial cells (Yung et al., 2009; Zhang et al., 2012). However, not all Abs to DNA share the same pathogenic potential. Certain subclasses of anti-double stranded (ds)DNA Abs are more closely associated with pathogenic potential, including human IgG1/IgG3 (Ravirajan et al., 2001), and mouse IgG2a in a murine lupus model (Savitsky et al., 2007). IgG subclass distribution of Abs to DNA differs between renal and extra-renal flares in patients with SLE (Bijl et al., 2002), while IgG3 deficient MRL/lpr mice have attenuated glomerulonephritis and prolonged survival (Greenspan et al., 2012). In addition, mice injected with monoclonal Abs (mAbs) with similar specificity for DNA but different IgG subclass and different relative affinity for basement membrane, showed unique patterns of IgG deposition in glomeruli and different levels of proteinuria (Krishnan et al., 2012). Therefore, the isotypes of Abs to DNA are apparently important in determining Ab pathogenic potential.

The Ig molecule consists of two identical heavy chains and two identical light chains, each of which has a variable (V) and a constant (C) region. Ab specificity was traditionally believed to be determined by the V regions, while the nature of the C region was responsible for Ab effector functions. During maturation of the murine immune response in secondary lymphoid organs, isotype switching can occur through an ordered sequence, generating Abs of different isotypes with presumably the same specificity because of unchanged V region sequences. Through in-vitro class switching, it is possible to generate a panel of isotype switched Abs which share the identical V region of the original Ab (Iglesias-Ussel et al., 2009).

In recent years, studies from several laboratories have demonstrated that the specificity and affinity of Abs are properties that may *not* be exclusively determined by the V region (Torres and Casadevall, 2008; Torres et al., 2007a; Dam et al., 2008; Torres et al., 2007b; Torres et al., 2005; Cooper et al., 1993; Pritsch et al., 2008; Tudor et al., 2012). Indeed, secondary structure changes occurring in Abs following antigen (Ag) binding can differ depending on the isotype (Janda and Casadevall, 2010). Furthermore, C regions can confer new properties to IgG molecules as seen by electronic emission spectra and proteolytic capacity (Janda et al., 2012). Therefore, at least in protective Abs the immunoglobulin C region may directly influence Ab-Ag interactions, possibly through alteration of Ab structure.

We recently generated a panel of murine anti-DNA Abs (IgG1, IgG2b, IgG2a, IgG3) which have identical V (variable-joining-diversity, or VDJ) regions. Members of this Ab panel not only differed in their affinity and specificity for nuclear Ags, but in binding to kidney Ags as well (Xia et al., 2012). Moreover, variations in affinity for renal Ags were mirrored in the analysis of specificity for glomeruli, and were associated with significant differences in renal pathogenicity and survival following Ab administration in-vivo. These results suggested that the C regions may be directly affecting the interaction between the Abs to DNA and their cognate Ags, and indicate that C region effects on Ab binding are directly relevant to understanding humoral autoimmunity as well.

How the C regions of anti-nuclear Abs affect binding to target Ags, however, remains unclear. The purpose of this study is to explore this observation and understand the relevant mechanisms, by investigating the effects of heavy chain C regions on the secondary structures of anti-nuclear Abs during their interaction with Ags.

2. Materials and methods

2.1. Generation of the PL9-11 isotype panel

As described previously (Xia et al., 2012), the murine IgG1, IgG2b and IgG2a isotype variants were generated from the parent hybridoma clone of PL9-11 (IgG3) by class switching in-vitro. All isotypes shared the original PL9-11 heavy and light chain V region, but differed in the identity of the heavy chain C region. The light chain variable-joining (VJ) sequence was published previously (GenBank: X65011.1). V region amino acid sequences are shown below (Trp residues indicated as “W”).

Heavy chain VDJ region—

EVKLVESGGGLVKPGGSLKLSCAASGFTFSNYIMFWVRQTPAKRLEWVANISSGGG
NTYYPD^{SVKGRFTISRDNVMN}ILYLQMS^{SLRSEDTAMYYCARRAYS}NYALDFW^{GQ}
GTSVTVS

Light chain VJ region—

IVMTQSPSSLSVSTGDKVTMSCKSSQSLLSNRNQN^{YLA}WYQQK^{PWQPPKLLIYGA}
STRKSGVPDRFTGSGSGTDFTLTISSVQTEDLAIYYC

(from beginning to end, underlined fragments are CDR1, CDR2 and CDR3, respectively).

To purify the mAbs, hybridoma cells were grown in a two-compartment tissue culture flask (Sartorius Co., Edgewood, NY). Supernatants were collected and passed over a protein A (IgG3, IgG2a) or protein G (IgG1, IgG2b) HiTrap column (GE Healthcare, Port Washington, NY). Purified Abs were then carefully normalized to the same Ab concentration by the shared kappa chain using both Western blotting and ELISA.

Coomassie blue staining performed on purified PL9-11 panel antibodies run on a protein gel demonstrated that the antibody preparations were free of significant histone contamination (data not shown).

2.2. Preparation of Ags

dsDNA was obtained from plasmid DNA grown in bacterial cells (which do not contain histones), by digesting with *XmnI*. Single stranded (ss) DNA was prepared by boiling dsDNA at 95°C for 5 min, followed by immediate cooling on ice. Histones H2A and H2B (DNA-free) were purchased from Roche (Pleasanton, CA). ssDNA, dsDNA, histone H2A and 2B all bound to the parent IgG3 mAb by ELISA and/or Westernblot (Xia et al., 2012).

2.3. SPR analysis

The affinity of the PL9-11 Abs for histone 2A and 2B was determined by SPR analysis using a Biacore 3000 instrument (Biacore, Piscataway, NJ). Ab concentration was titrated from 0–200 nM in running buffer (pH 7.4, 20 mM KPO₄, 130 mM KCl, 3.4 mM EDTA and 0.005% Tween 20). Histones 2A or 2B (5 nM) were immobilized on a CM sensor chip (GE Healthcare). The Abs were injected over the chip at a flow rate of 30 µl per min, with 280 seconds for contact time and 350 seconds for dissociation. All responses were normalized and expressed relative to the baseline defined by the running buffer. The simple Langmuir model ($A + B \leftrightarrow AB$) was used for calculating the binding kinetics of the Ab-Ag complex. Association (K_a) and dissociation (K_d) values were calculated using the Biacore evaluation software 1.1 (Hedberg et al., 2011).

2.4. Trp fluorescence emission

The FluoroMax-3 spectrofluorometer (HORIBA Jobin Yvon, Stanmore Middlesex, UK) was utilized to measure the intensity of fluorescence at a wavelength range of 295 to 450 nm. The Ab panel, Ags (dsDNA, ssDNA, histone 2A and 2B), and their complexes were prepared at room temperature by mixing the two components 30 min before recording the spectra. All the Ags and Abs were mixed at same final molar concentration of 0.5 μ M. Three independent repeats were performed for each experimental condition.

For salt titration, PL9-11 IgG3 and dsDNA (0.5 μ M) were solubilized in a NaCl solution that had a concentration of 25, 50, 100, 200, and 400 mM. The final data was recorded after subtracting the spectra of the NaCl solution alone.

2.5. CD spectroscopy

CD spectroscopy was carried out using a Jasco spectrophotometer (JASCO, Easton, MD) (Janda and Casadevall, 2010), with a 350 μ l rectangular quartz cuvette for the samples. ssDNA and dsDNA were prepared at final concentrations of 2.5, 5, 10, 20, 40 and 80 nM in PBS. Histones H2A and H2B were used at 80, 90, 100, 110, 120 and 130 nM. Ab concentrations were kept constant at 80 nM. Data were measured from a range of 240 nm to 190 nm. After converting machine units θ_{obs} (millidegrees) to delta epsilon ($\Delta\epsilon$), the secondary structure prediction was done by using the CONTIN/LL program in the CDPro suite (Janda and Casadevall, 2010). The reference set of protein provided the structures of regular α helix (Hr), distorted α helix (Hd), regular β sheet (Sr), distorted β sheet (Sd), turns (Turn), and unordered (Unrd).

2.6. Control proteins

Murine mAb isotype controls were obtained from Southern Biotech (Birmingham, AL): IgG3, B10 (Ag unknown); IgG1, 15H6 (T-2 mycotoxin); IgG2b, A-1 (chicken IgA); IgG2a, HOPC-1 (unknown). All of the isotype controls were verified not to bind to dsDNA. Murine IL-4 (Cell Signaling, Danvers, MA) was used as the Ag control for histones 2A and 2B (m.w. 14 kDa) because of a similar molecular weight (16 kDa), and the absence of Trp in the amino acid sequence.

2.7. Statistical analysis

All results were shown as mean $\pm$ SEM. ANOVA analysis of variance was used for the comparison of more than two groups. A two-tailed *t*-test was used when two groups were compared for statistical differences. Spearman's rank correlation coefficients were computed to investigate the correlations between NaCl concentrations and peak wavelength of Trp fluorescence or K_D values in salt titration. *P* values less than 0.05 were considered significant.

3. Results

3.1. PL9-11 isotype variants exhibit different affinity to histone Ags

Surface plasmon resonance (SPR) was used to measure the affinity of the PL9-11 Ab panel to histones 2A and 2B. We found that members of the PL9-11 Ab panel had different binding kinetics when reacting with histone 2A or 2B (Fig. 1, Table 1). The response signal increased in a dose-dependent manner, with the highest value seen in the IgG2a isotype (Fig. 1). The relative affinities of the other isotypes for histone 2A (compared to IgG2a which was normalized to a value of 1) were 0.48 (IgG3), 0.19 (IgG1), and 0.09 (IgG2b). For histone 2B, the relative affinities (similarly compared to IgG2a) were 0.56 (IgG3), 0.23 (IgG1), and 0.13 (IgG2b), respectively.

To verify that our results reflect true binding to histone and that the antibody preparations were free of DNA, we repeated the SPR binding analysis with and without antibody (PL9-11 IgG2a and IgG2b) pretreatment with DNase. The K_D values and SPR curves reflecting binding of these isotypes to histone 2A were not significantly altered (data not shown). Similarly, no DNA was visualized in the histone preparations following propidium iodide treatment (data not shown). Furthermore, using DNase treated histones as a source of antigen did not result in any significant differences in antibody binding to histone 2A and 2B by ELISA (data not shown), indicating that the histone preparations were indeed DNA free.

3.2. Fluorescence emission spectra reveal changes in Ab structure upon Ag binding

Since the PL9-11 derived mAbs had identical V region sequences, we hypothesized that the Abs would have similar fluorescence emission spectral changes upon binding to Ag if the C regions have indeed no effect on Ag binding. The amino acid sequences of the PL9-11 clones have ten tryptophan (Trp) residues in the V region of each Ab, six in the heavy chain and four in the light chain. There is one Trp residue neighboring CDR1 and one Trp residue neighboring CDR3 in the heavy chain, and one neighboring CDR1 in the light chain (Losman et al., 1993), which may be involved in the Ag-Ab interaction.

We found that members of the PL9-11 panel differed in their baseline Trp fluorescence, as would be expected for Abs with different numbers of Trp and tyrosine residues. Peaks of emission spectra were at 343 nm, 335 nm, 336 nm, and 339 nm for IgG3, IgG1, and IgG2b respectively. For all Abs, Ag binding resulted in either a blue (left) or red (right) shift in the maximal value of Trp emission depending on the isotype and Ag (Fig. 2). Binding to ssDNA, the PL9-11 IgG3 and IgG2a mAbs showed red shifting, or increased polarity; in contrast, IgG1 and IgG2b displayed the reverse (blue shift) changes, indicating decreased polarity. With binding to dsDNA, all isotypes displayed red shifting of the Trp emission peaks. For the histone Ags, the direction and/or magnitude of curve shifts also differed among the isotypes. IgG3 had the largest magnitude of change after binding to histone 2A, while IgG1 was the isotype with the most prominent shift upon histone 2B binding. Significant differences were found between IgG3 and IgG1 in the extent of wavelength changes following Ag binding, for all 4 Ags tested (Table 2). Depending on the specific Ag and isotype, other comparisons were significant as well (Table 2). The magnitude of changes seen in the Trp emission wavelengths, and how much they differed among the various isotypes, is consistent with and supportive of a conclusion that these isotypes differ in their paratope (e.g. the antigen binding surface of the Ab) as a result of their adjoining C region types.

We also compared the fluorescence intensity at the peak emission (Fig. 2, y-axis) of the Ab alone, to the samples of Ab+Ag. Significant differences ($p < 0.05$) were found for IgG3, IgG2b, and IgG2a binding to dsDNA, IgG3 binding to ssDNA, and IgG2a binding to histone 2A (Fig. 2).

3.3. Ag-anti-nuclear Ab binding is partially electrostatic

To further explore the nature of the anti-DNA Ab-dsDNA binding interaction and determine whether it is salt dependent, we repeated the Trp fluorescence experiments while varying the concentration of NaCl. Increasing the salt concentration in the solution for dsDNA binding to the PL9-11 IgG3 mAb resulted in blue shifting of the Trp emission curve, as the NaCl concentration was gradually increased from 25 to 400 mM (Fig. 3A). The wavelength at the peak of fluorescence decreased from 347 nm at 25 mM to 335 nm at 400 mM NaCl (Fig. 3B), indicating a decrease in polarity.

We repeated the salt titration analysis using SPR to analyze PL9-11 mAbs binding to histones. We found that for the same PL9-11 IgG2a concentration, the response signal increased with higher NaCl concentrations (Fig. 4A). Interestingly, for histone 2A and 2B the shape of the signal curves were similar even as NaCl concentration changed, but differed between these two related Ags (Fig. 4A, 4B). The calculated K_D values decreased with increasing salt concentrations (Fig. 4B).

3.4. The secondary structure content of Abs is altered upon Ag binding

The secondary structure content of members of the PL9-11 mAb panel alone, and in complex with Ag, was measured by circular dichroism (CD) spectroscopy. At baseline, before the addition of Ags, the different PL9-11 isotypes showed significant differences in their native secondary structures. The native structures differed most between the IgG3/IgG2a isotypes and the IgG2b/IgG1 isotypes (Fig. 5A). The regular β sheet (Sr), distorted β sheet (Sd), turns (Turn), and unordered (Unrd) secondary structure estimates dominated the secondary structures of these Abs (Fig. 5B). Upon complex formation (molar ratio of Ag:Ab = 1:1), the Ab secondary structure content changed, and these changes differed among the isotypes. When the Abs were complexed with DNA, the addition of dsDNA (Fig. 5C) or ssDNA (Fig. 5D) caused significant changes in secondary structure content in all of the isotypes ($p < 0.05$). Similar results were observed when the Ag used was ssDNA. Therefore, for each isotype, the binding to DNA affected the secondary structures differently. Furthermore, the effects of Ag binding on Ab secondary structure were Ag dependent. Importantly, the absence of histone contamination of the DNA used in these studies was confirmed by the lack of changes in antibody binding to DNA following proteinase K treatment of the DNA antigen (data not shown).

The changes observed with CD spectroscopy using various molar ratios of the PL9-11 IgG3 dsDNA Ab plateaued at Ab:Ag molar ratios of 1:3.2 (Fig. 6). Binding to ssDNA Ag was analyzed at a similar range of Ab:Ag ratios, and saturation was reached later, at approximately 1:3.3 (not shown). Similar results were seen for all of the PL9-11 isotypes (not shown). On the other hand, the optimal molar ratios for histone Ag:Ab concentrations before secondary structure changes reached a plateau were 1:0.71 for histone 2A and 1:0.78 for histone 2B (not shown).

Since histones 2A and 2B have protein secondary structures that contribute to the CD spectra of the Ag-Ab complex, their endogenous signal needed to be taken into consideration. Consequently, CD spectroscopy was performed to analyze complexes of Ab plus either of the histone Ags. Murine IL-4, which has its own native secondary structure, was utilized as an Ag control for comparison. IL-4 was specifically chosen since it has similar molecular weight to histones 2A and 2B, yet it does not bind to members of the PL9-11 mAb panel. We found that the secondary structure content in the samples containing the individual PL9-11 isotypes and IL-4 was always within the range of values found for Ab alone and IL-4 alone (Fig. 7). However, the secondary structure content of the samples containing PL9-11 mAb and histone did not follow this rule. For example, the Sd and Unrd contents of the IgG2a plus histone 2A were higher and lower, respectively, than those of either IgG2a or histone 2A alone ($p < 0.05$). Similar results were seen with histone 2A and 2B in other isotypes as well.

We also analyzed the CD spectrum of the non-nuclear Ag binding isotype controls. As we found with the samples containing the individual PL9-11 mAbs together in solution with an Ag they do not bind to, the values for the structural content of isotype controls (which do not bind histones) plus histone consistently fell within the range defined by Ab alone and Ag alone (data not shown).

4. Discussion

In this study we demonstrate that anti-nuclear Abs with identical V regions but different C regions exhibit diverse binding affinities to histone. Although these observations could reflect differences in accessibility to binding sites depending on the flexibility of the various Fab in association with different C regions, our work also strongly implies differences at the paratope level. Specifically, we found that the interaction between PL9-11 mAbs and nuclear Ags resulted in a shift in the peak emission wavelength of Trp fluorescence as compared to Abs alone. The direction and magnitude of this shift was isotype and Ag dependent. In addition, the secondary structure content in PL9-11 isotypes showed significant changes upon formation of Ag:Ab complexes with DNA and histone Ags. It remains to be seen whether these structural changes occurring in the antigen binding surface only affect the chemistry of binding, leaving the cognate antigen epitope unchanged but changing the affinity of the interaction, or whether actual epitope drift is occurring. In contrast, adding an Ag to which the Abs did not bind did not affect secondary structures. Similarly, secondary structure components of isotype control Abs were not influenced by the presence of DNA or histone. Taken together, these studies demonstrate that the C region plays an important role in the affinity and specificity of anti-nuclear Abs.

Our results are consistent with and supportive of the finding by Janda et al. that isotype-switched anti-glucuronoxylomannan (1,200 kDa) Abs express different paratopes (Janda et al., 2012). By utilizing nuclear magnetic resonance spectroscopy, Janda et al. reported that the C region imposes conformational constraints on the V region to affect the paratope structures in anti-glucuronoxylomannan Abs (Janda et al., 2012). Other Ags such as N-acetyl-glucosamine-bovine serum albumin (120 kDa) (Cooper et al., 1993), β -tubulin (54 kDa) (Pritsch et al., 2000), HIV-1 gp41 (21 kDa) (Tudor et al., 2012), and anthrax (Abboud et al., 2010) were used in comparable analyses. These studies have similarly found specificity differences among IgG Abs expressing identical variable domains, albeit not in all cases (Abboud et al., 2010). Nevertheless, our studies are the first to demonstrate a similar phenomenon for pathogenic autoantibodies, and have important implications in understanding systemic autoimmunity. Furthermore, our observations may shed light on the mechanism by which certain anti-DNA Abs are cross-reactive with structural Ags, and why immunoglobulin deposition in target organs can be isotype specific (Savitsky et al., 2010). Additional studies will be needed however to determine if isotype-dependent changes in specificity are ubiquitous in all Ab/Ag systems, in which case it will be important to understand how these are handled by the immune system. The alternative possibility is that this phenomenon is restricted to a more limited set of antibodies with defined specificities, which would explain why specific isotypes are associated with certain disease states.

In the present study, both protein (histone) and non-protein (DNA) Ags were used in the binding analyses. Histone 2A and 2B have a molecular weight of 14 kDa, and have no Trp residues. The dsDNA (296 kDa) and ssDNA (148 kDa) Ags were derived from plasmid DNA. It has already been demonstrated previously that cross-reactive antigens do not have to be structurally homologous (Gaynor et al., 1997). Nevertheless, it is important to try to understand how a single monoclonal antibody can bind such disparate antigens. We hope to be able to address this question directly in future studies by careful analysis of antibody crystal structure. Nonetheless, based on these and previous studies, we can conclude that Ab binding to a variety of Ags with quite different molecular weights and biochemical properties (polysaccharide, protein, or nucleic acid) can all affect the Ab structure.

SPR analysis revealed differential binding affinity of the PL9-11 isotypes Abs to both histone 2A and 2B in order of IgG2a>IgG3>IgG1>IgG2b, while we determined previously that the relative affinity of these mAbs for dsDNA was IgG3>IgG2a>IgG1>IgG2b (Xia et

al., 2012). Abs both to DNA and histone are believed to be important in the pathogenesis of SLE (Muller et al., 2008; Kiss et al., 2009; Cohen-Solal and Diamond, 2011; Pavlovic et al., 2011), and at least for pathogenic anti-DNA Abs there is a bias towards Abs of the IgG2a and IgG3 isotypes (Sugiyama et al., 2004; Baudino et al., 2006; Mannoor et al. 2012; Moser et al., 2012). It is important to note that variations in binding between PL9-11 switch variants were not only related to C region differences, but also to the properties of the Ag studied. Furthermore, we found a significant difference between the SPR signal curves of histone 2A and 2B, with higher relative stability for PL9-11-histone 2A interactions.

Trp fluorescence is a powerful tool for structural analysis of proteins that have endogenous Trp residues; the technique is non-destructive and does not involve any externally introduced perturbations. Fluorescence from a specific Trp residue is extremely sensitive to the polarity of its environment (Sun et al., 2010); hence, it is an excellent reporter for structural changes arising either due to internal dynamics of the protein, or ligand binding that alters the environment. Due to the aromatic character of Trp residues, they are in general found fully or partially buried in the hydrophobic core or patches of the proteins with distinct fluorescence emission wavelengths, i.e. the more blue shifted the more buried the residue is (Gensch et al., 2004; Dasgupta et al., 2012). Thus, Trp residues in and/or close to the CDR loops are excellent tools for probing Ag-Ab interactions.

An Ab interaction with its cognate Ags leads to changes of peak emission wavelength in Trp fluorescence. Left, or blue-shifting, of the emission peak indicates a less polar Trp environment in the complexes, while right, or red-shifting, suggests a more polar Trp environment. The Ag-Ab interaction involves and often changes the local environments of the CDR loops; hence, the environment of the Trp residues adjacent to the CDR loops can be affected as well. Our results showed that Trp emission significantly changed upon Ag binding. Depending on the particular Ab-Ag pair, Trp emission was either blue or red shifted compared to unbound Ab, indicating differences in Ab-Ag binding mechanisms dependent on both isotype and Ag properties. The IgG3 isotype had the largest magnitude of shift after binding to dsDNA (red), ssDNA (red), and histone 2A (blue). In contrast, for histone 2B, IgG1 gave the most significant shift (red). The hinge regions of the four IgG isotypes do not contain Trp (not shown); although the C regions do contain Trp residues with some differences between isotypes, the Trp residues that shift are most reasonably those in the closest proximity to the antigen binding surface. Therefore, the majority of the signal we observed in these experiments is likely coming from the V region. These results point to the role of the C region in modulating the fine specificity of Abs, through changes in the local electronic environments of their paratopes.

In some of the Trp emission panels, the addition of Ags not only causes a shift in emission wavelength, but the signal also manifested significantly altered intensity. This may be caused by various quenching groups that come closer to, or move further away from, the Trp residues depending on the mechanism of the Ab:Ag interaction. Several amino-acid residues and peptide bonds are known to be efficient quenchers of Trp fluorescence. These quenchers influence the Trp residues through excited-state electron/proton transfers, causing a decrease in Trp fluorescence quantum yield (Adams et al., 2002; Kurz et al., 2005). The fluorescence intensity changes indicate that an interaction is occurring between Ab and Ag; accordingly, the differences in intensity shifts point toward a conclusion that members of the PL9-11 mAb panel differ in how they interact with Ag, which is consistent with differences in binding site structure despite identical V regions.

Our experiments with NaCl titrations revealed that Trp fluorescence could be affected by changing ionic strength. This effect can be explained by assuming that the interaction between Ab and Ag is electrostatic to some extent, allowing NaCl to compete with Ag thus

affecting Trp fluorescence. If increasing NaCl concentration induces tighter Ag:Ab binding resulting in further burial of Trp residues and excluding solvent molecules, this would decrease the polarity of the interaction and subsequently results in a blue shift in fluorescence emission. Salt titration was performed for the SPR analysis as well, and showed that binding affinity for the IgG2a-histone pairs was inversely related to salt concentrations. Lower K_D values (higher affinity) may arise either due to the weakening of repulsive electrostatic forces between Ag and Ab or due to the increased hydrophobicity at higher salt concentrations. Therefore, these results are consistent with effect of NaCl titrations shown in Trp fluorescence. Interestingly, for a given histone Ag, the shapes of signal curves in the SPR salt titration were similar when NaCl concentration was altered, indicating that the binding behavior of Ab to histone Ags does not change as a function of NaCl content.

CD spectroscopy measures the secondary structures of protein by detecting differences in the absorption of left and right handed circularly polarized light using the far ultraviolet absorption spectra (Kelly and Price, 2000). Similar to Trp fluorescence, the CD analysis exhibited changes in both direction and magnitude that differed among the PL9-11 isotypes upon interaction with Ag. Relatively small changes were seen percentage wise in the CD spectroscopy experiments, accounting for alterations in a few residues, yet these were sufficient for measurable changes in affinity and specificity. One caveat in the interpretation of these changes is that Ab binding to the protein Ag could induce a structural change in the latter that lead to CD changes. This effect would apply to histones but not to DNA.

It is important to consider whether hinge structures, which differ between the isotypes, may be contributing to the CD changes observed. However, if the hinge region is dynamic in solution, this would usually be manifest in the “turn” or “undefined” categories, and their secondary structure may not be well defined by this technique. Taking into consideration the Trp fluorescence data as well, since the PL9-11 isotypes share identical V regions the most reasonable conclusion from the totality of results presented here is therefore that C regions have differential effects on autoantibody V region secondary structures after binding to DNA and histone that influence Ag-Ab complex formation. Although this will need to be demonstrated experimentally, we speculate that these sometimes subtle structural changes may further translate into significant consequences in nephritogenic potential. Indeed, the fact that the IgG2a isotype displayed relatively more frequent changes in secondary structures following autoAg binding may at least partially explain the association of this isotype in active murine lupus, and particularly renal disease (Baudino et al., 2006).

Analysis of the PL9-11 panel of mAbs in this study, with different heavy chains and constant light chains, revealed significant changes in several parameters reflecting Ab structure. These observations are consistent with previous studies by Stollar et al. which demonstrated the dominant role of the heavy chain in the binding of DNA Ag, although the light chain does add slightly to the binding energy (Polymenis and Stollar, 1995; Jang et al., 1996; Chen and Stollar, 1999). Furthermore, Sela-Culang et al. recently undertook a systematic analysis of all Ab crystal structures that were solved in both their free and Ag-bound form. Among the conformational changes that are reported to occur following Ag binding, the most significant and frequent ones occur in the C_H region (Sela-Culang et al., 2012). Nevertheless, although outside the scope of the present study, it is possible that switching a lambda with a kappa light chain may have induced similar changes to those brought on by heavy chain isotype switching. Another important observation from this group is that besides their actual sequences and length, the binding affinities of anti-DNA Abs to DNA Ags depends largely on the structure of oligonucleotides (Stollar, 1997; Stollar, 1989; Stollar, 1994; Edginton and Stollar, 1992). Our results presented above are therefore complementary, showing that structural variations in either the anti-nuclear Ab (current

study) or its cognate Ag (Stollar et al.) are instrumental in determining the actual affinity of the interaction between DNA and anti-DNA Abs.

In conclusion, our results provide convincing evidence that the C region affects the binding of anti-nuclear Abs to DNA and histone Ags by altering the local electronic environments of their paratopes, as well as their secondary structures. Hence, we extend this phenomenon to the realm of autoantibodies. C regions neighboring the V region therefore confer novel properties to lupus-associated autoantibodies, which manifest in the electronic emission spectra. Finally, these studies suggest that the mechanism by which the C region may contribute to the antigenic specificity and renal pathogenicity of murine anti-nuclear Abs is through its allosteric influence on the V region.

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Abbreviations

Ag	Antigen
Ab	antibody, C, constant
CD	circular dichroism
ds	double stranded
mAb	monoclonal antibody
Sd	distorted β sheet
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
Sr	regular β sheet
ss	single stranded
Trp	tryptophan
Turn	turns
Unrd	unordered
V	variable
VDJ	variable-joining-diversity

References

- Abboud N, Chow SK, Saylor C, Janda A, Ravetch JV, Scharff MD, Casadevall A. A requirement for Fc γ R in antibody-mediated bacterial toxin neutralization. *J Exp Med*. 2010; 11:2395–2405. [PubMed: 20921285]
- Adams PD, Chen Y, Ma K, Zagorski MG, Sönnichsen FD, McLaughlin ML, Barkley MD. Intramolecular quenching of tryptophan fluorescence by the peptide bond in cyclic hexapeptides. *J Am Chem Soc*. 2002; 124:9278–9286. [PubMed: 12149035]

- Baudino L, Azeredo da Silveira S, Nakata M, Izui S. Molecular and cellular basis for pathogenicity of autoantibodies: lessons from murine monoclonal autoantibodies. *Springer Semin Immunopathol.* 2006; 28:175–184. [PubMed: 16953439]
- Bijl M, Dijkstra HM, Oost WW, Bootsma H, Derksen RH, Aten J, Limburg PC, et al. IgG subclass distribution of autoantibodies differs between renal and extra-renal relapses in patients with systemic lupus erythematosus. *Rheumatology.* 2002; 41:62–67. [PubMed: 11792881]
- Chen Y, Stollar BD. DNA binding by the VH domain of anti-Z-DNA antibody and its modulation by association of the VL domain. *J Immunol.* 1999; 162:4663–4670. [PubMed: 10202006]
- Cohen-Solal J, Diamond B. Lessons from an anti-DNA autoantibody. *Mol Immunol.* 2011; 48:1328–1331. [PubMed: 21216012]
- Cooper LJ, Shikhman AR, Glass DD, Kangisser D, Cunningham MW, Greenspan NS. Role of heavy chain constant domains in antibody-antigen interaction. Apparent specificity differences among streptococcal IgG antibodies expressing identical variable domains. *J Immunol.* 1993; 150:2231–2242. [PubMed: 7680687]
- Dam TK, Torres M, Brewer CF, Casadevall A. Isothermal titration calorimetry reveals differential binding thermodynamics of variable region-identical antibodies differing in constant region for a univalent ligand. *J Biol Chem.* 2008; 283:31366–31370. [PubMed: 18806257]
- Dasgupta A, Udgaonkar JB. Transient non-native burial of a Trp residue occurs initially during the unfolding of a SH3 domain. *Biochemistry.* 2012; 51:10210. [PubMed: 22100862]
- Edgington SM, Stollar BD. Immunogenicity of Z-DNA depends on the size of polynucleotide presented in complexes with methylated BSA. *Mol Immunol.* 1992; 29:609–617. [PubMed: 1584229]
- Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD, Diamond B. Peptide inhibition of glomerular deposition of an anti-DNA antibody. *Proc Natl Acad Sci U S A.* 1997; 94:1955–60. [PubMed: 9050886]
- Gensch T, Hendriks J, Hellingwerf KJ. Tryptophan fluorescence monitors structural changes accompanying signalling state formation in the photocycle of photoactive yellow protein. *Photochem Photobiol Sci.* 2004; 3:531–536. [PubMed: 15170481]
- Greenspan NS, Lu MA, Shipley JW, Ding X, Li Q, Sultana D, Kollaros M, et al. IgG3 deficiency extends lifespan and attenuates progression of glomerulonephritis in MRL/lpr mice. *Biol Direct.* 2012; 7:3. [PubMed: 22248284]
- Hedberg A, Fisman S, Fenton KA, Fenton C, Osterud B, Mortensen ES, Rekvig OP. Heparin exerts a dual effect on murine lupus nephritis by enhancing enzymatic chromatin degradation and preventing chromatin binding in glomerular membranes. *Arthritis Rheum.* 2011; 63:1065–1075. [PubMed: 21190297]
- Iglesias-Ussel MD, Zavadi J, Scharff MD. Molecular characterization of hybridoma subclones spontaneously switching at high frequencies in-vitro. *J Immunol Methods.* 2009; 350:71–78. [PubMed: 19619554]
- Janda A, Casadevall A. Circular Dichroism reveals evidence of coupling between immunoglobulin constant and variable region secondary structure. *Mol Immunol.* 2010; 47:1421–1425. [PubMed: 20299100]
- Janda A, Eryilmaz E, Nakouzi A, Cowburn D, Casadevall A. Variable region identical immunoglobulins differing in isotype express different paratopes. *J Biol Chem.* 2010; 285:35409–35417. [PubMed: 22930758]
- Jang EJ, Nahm DH, Jang YJ. Mouse monoclonal autoantibodies penetrate mouse macrophage cells and stimulate NF-kappaB activation and TNF-alpha release. *Immunol Lett.* 2009; 124:70–76. [PubMed: 19394364]
- Jang YJ, Lecerf JM, Stollar BD. Heavy chain dominance in the binding of DNA by a lupus mouse monoclonal autoantibody. *Mol Immunol.* 1996; 33:197–210. [PubMed: 8649441]
- Kelly SM, Price NC. The use of circular dichroism in the investigation of protein structure and function. *Curr Protein Pept Sci.* 2000; 1:349–384. [PubMed: 12369905]
- Kiss E, Lakos G, Szegedi G, Poor G, Szodoray P. Anti-nucleosome antibody, a reliable indicator for lupus nephritis. *Autoimmunity.* 2009; 42:393–398. [PubMed: 19811255]

- Krishnan MR, Wang C, Marion TN. Anti-DNA autoantibodies initiate experimental lupus nephritis by binding directly to the glomerular basement membrane in mice. *Kidney Int.* 2012; 82:184–192. [PubMed: 22297676]
- Kurz LC, Fite B, Jean J, Park J, Erpelding T, Callis P. Photophysics of tryptophan fluorescence: link with the catalytic strategy of the citrate synthase from *Thermoplasma acidophilum*. *Biochemistry.* 2005; 44:1394–1413. [PubMed: 15683225]
- Losman MJ, Fasy TM, Novick KE, Monestier M. Relationships among antinuclear antibodies from autoimmune MRL mice reacting with histone H2A–H2B dimers and DNA. *Int Immunol.* 1993; 5:513–523. [PubMed: 8318454]
- Mannoor K, Matejuk A, Xu Y, Beardall M, Chen C. Expression of natural autoantibodies in MRL-lpr mice protects from lupus nephritis and improves survival. *J Immunol.* 2012; 188:3628–3638. [PubMed: 22407922]
- Moser K, Kalies K, Szyska M, Humrich JY, Amann K, Manz RA. CXCR3 promotes the production of IgG1 autoantibodies but is not essential for the development of lupus nephritis in NZB/NZW mice. *Arthritis Rheum.* 2012; 64:1237–1246. [PubMed: 22006377]
- Muller S, Dieker J, Tincani A, Meroni PL. Pathogenic anti-nucleosome antibodies. *Lupus.* 2008; 17:431–436. [PubMed: 18490422]
- Pavlovic M, Kats A, Cavallo M, Chen R, Hartmann JX, Shoenfeld Y. Pathogenic and epiphenomenal anti-DNA antibodies in SLE. *Autoimmune Dis.* 2010;462841. 2011. [PubMed: 21152217]
- Polymenis M, Stollar BD. Domain interactions and antigen binding of recombinant anti-Z-DNA antibody variable domains. The role of heavy and light chains measured by surface plasmon resonance. *J Immunol.* 1995; 154:2198–2208. [PubMed: 7868893]
- Pritsch O, Magnac C, Dumas G, Bouvet JP, Alzari P, Dighiero G. Can isotype switch modulate antigen-binding affinity and influence clonal selection? *Eur. J Immunol.* 2000; 30:3387–3395.
- Qing X, Pitashny M, Thomas DB, Barrat FJ, Hogarth MP, Putterman C. Pathogenic anti-DNA antibodies modulate gene expression in mesangial cells: involvement of HMGB1 in anti-DNA antibody-induced renal injury. *Immunol Lett.* 2008; 121:61–73. [PubMed: 18822317]
- Ravirajan CT, Rowse L, MacGowan JR, Isenberg DA. An analysis of clinical disease activity and nephritis-associated serum autoantibody profiles in patients with systemic lupus erythematosus: a cross-sectional study. *Rheumatology.* 2001; 40:1405–1412. [PubMed: 11752513]
- Savitsky DA, Yanai H, Tamura T, Taniguchi T, Honda K. Contribution of IRF5 in B cells to the development of murine SLE-like disease through its transcriptional control of the IgG2a locus. *Proc Natl Acad Sci U S A.* 2010; 107:10154–10159. [PubMed: 20479222]
- Sela-Culang I, Lon AS, Ofra Y. A systemic comparison of free and bound antibodies reveals binding-related conformational changes. *J Immunol.* 2012; 189:4890–4899. [PubMed: 23066154]
- Stollar BD. Molecular analysis of anti-DNA antibodies. *FASEB J.* 1994; 8:337–342. [PubMed: 7511550]
- Stollar BD. Why the difference between B-DNA and Z-DNA? *Lupus.* 1997; 6:327–328. [PubMed: 9296781]
- Stollar BD. Immunochemistry of DNA. *Int Rev Immunol.* 1989; 5:1–22. [PubMed: 2491157]
- Sugiyama M, Funauchi M, Yamagata T, Nozaki Y, Yoo BS, Ikoma S, Kinoshita K, et al. Predominant inhibition of Th1 cytokines in New Zealand black/white F1 mice treated with FK506. *Scand J Rheumatol.* 2004; 33:108–114. [PubMed: 15163112]
- Sun F, Zong W, Liu R, Chai J, Liu Y. Micro-environmental influences on the fluorescence of tryptophan. *Spectrochim Acta A Mol Biomol Spectrosc.* 2010; 76:142–145. [PubMed: 20363661]
- Torres M, Casadevall A. The immunoglobulin constant region contributes to affinity and specificity. *Trends Immunol.* 2008; 29:91–97. [PubMed: 18191616]
- Torres M, Fernandez-Fuentes N, Fiser A, Casadevall A. The immunoglobulin heavy chain constant region affects kinetic and thermodynamic parameters of antibody variable region interactions with antigen. *J Biol Chem.* 2007a; 282:13917–13927. [PubMed: 17353196]
- Torres M, Fernandez-Fuentes N, Fiser A, Casadevall A. Exchanging murine and human immunoglobulin constant chains affects the kinetics and thermodynamics of antigen binding and chimeric antibody autoreactivity. *PLoS One.* 2007b; 2:e1310. 2007. [PubMed: 18074033]

- Torres M, May R, Scharff MD, Casadevall A. Variable-region-identical antibodies differing in isotype demonstrate differences in fine specificity and idiotype. *J Immunol.* 2005; 174:2132–2142. [PubMed: 15699144]
- Tudor D, Yu H, Maupetit J, Drillet AS, Bouceba T, Schwartz-Cornil I, et al. Isotype modulates epitope specificity, affinity, and antiviral activities of anti-HIV-1 human broadly neutralizing 2F5 antibody. *Proc Natl Acad Sci U S A.* 2012; 109:12680–12685. [PubMed: 22723360]
- Xia Y, Pawar RD, Nakouzi AS, Herlitz L, Broder A, Liu K, Goilav B, et al. The constant region contributes to the antigenic specificity and renal pathogenicity of murine anti-DNA antibodies. *J Autoimmun.* 2012; 39:398–411. [PubMed: 22841793]
- Yung S, Chan TM. Anti-DNA antibodies in the pathogenesis of lupus nephritis--the emerging mechanisms. *Autoimmun Rev.* 2008; 7:317–321. [PubMed: 18295737]
- Yung S, Tsang RC, Sun Y, Leung JK, Chan TM. Effect of human anti-DNA antibodies on proximal renal tubular epithelial cell cytokine expression: implications on tubulointerstitial inflammation in lupus nephritis. *J Am Soc Nephrol* 2005. 2005; 16:3281–3294.
- Yung S, Zhang Q, Zhang CZ, Chan KW, Lui SL, Chan TM. Anti-DNA antibody induction of protein kinase C phosphorylation and fibronectin synthesis in human and murine lupus and the effect of mycophenolic acid. *Arthritis Rheum.* 2009; 60:2071–2082. [PubMed: 19565476]
- Zhang Y, Yang J, Jiang S, Fang C, Xiong L, Cheng H, Xia Y. The lupus-derived anti-double-stranded DNA IgG contributes to myofibroblast-like phenotype in mesangial cells. *J Clin Immunol.* 2012; 32:1270–1278. [PubMed: 22730054]

Highlights

1. Autoantibody isotype switch variants display significant differences in histone affinity;
2. Tryptophan fluorescence emission profiles were isotype dependent;
3. Changes in antibody secondary structure content differed between isotypes upon antigen binding;
4. C regions neighboring the V region confer novel properties to autoantibodies

Fig. 1. SPR analysis of the binding affinity of PL9-11 isotypes to histones. Histone 2A or 2B (5 nM) were immobilized on the sensor chip, followed by running the PL9-11 isotype at 0, 20, 40, 80, 120, and 200 nM. The sensorgrams depicted in this figure display histone binding to Abs at 40 nM. The results in this figure are representative of three independent repeats.

Fig. 2.

Trp fluorescence of PL9-11 mAbs before and after binding to Ag. The excitation wavelength was 285 nm. The arrowheads point to the peaks of the emission curves, and the long horizontal arrows indicate the directions of the shifts. Results depicted in this figure are representative of three independent experiments.

Fig. 3.

Trp fluorescence of PL9-11 IgG3 at different NaCl concentrations. (A) The binding curve for PL9-11 IgG3 binding to dsDNA shifted to the left with increasing NaCl concentrations. (B) The wavelength of the Trp fluorescence peak gradually decreased as NaCl concentrations in the solution increased ($p < 0.05$, $n = 3$ for each concentration).

Fig. 4.

The effect of NaCl titration on SPR analysis of PL9-11 IgG3 binding to histone. Histone 2A or 2B at 5 nM were immobilized to the sensorchip, and the PL9-11 IgG3 (0–200 nM) was solubilized in a range of NaCl concentrations. (A) Representative sensorgrams demonstrate that the response signal of Ab (at 40 nM) binding to histones increases with rising salt concentrations. (B) The K_D values of histone binding were calculated based on the data from six different PL9-11 IgG3 concentrations, and exhibit a declining trend as NaCl concentrations increase ($p < 0.05$, $n = 3$ at each concentration).

Fig. 5. CD analysis of Abs alone, and in complex with Ag. The representative histograms of Abs alone (80 nM) were shown after the subtraction of buffer signal. The final concentration of DNA was 80 nM. H(r) = regular α helix; H(d) = distorted α helix; S(r) = regular β strand; S(d) = distorted β strand; Turn = turns; Unrd = unordered. * = $p < 0.05$, compared to the PL9-11 IgG3 isotype. Δ = $p < 0.05$, compared to the non-bound state. Three independent repeats were performed for each sample, with similar results.

Fig. 6. Changes in secondary structure content in the PL9-11 mAb panel when binding to dsDNA, at different Ag-Ab ratios. The concentrations of dsDNA were set at 0, 2.5, 5, 10, 20, 40 and 80 nM for the CD spectroscopy, while Ab concentration was kept constant at 80 nm. The concentration of Ag at which the secondary structure curve for each component appears to plateau is denoted with a vertical line.

Fig. 7.

Secondary structure content of Ags alone, PL9-11 mAbs alone, and their mixture. The results of three independent repeats using histones and a control Ag (murine IL-4) are shown, with Ab at 80 nM and Ag at 130 nM. Secondary structural content of samples of PL9-11 plus IL-4 were consistently within the ranges found for Ab or Ag alone. Conditions in which secondary structure content exceeded these ranges in mixtures of PL9-11 mAb plus histone are marked with an asterisk.

Table 1

Binding affinity of the different PL9-11 isotypes to histones.

Antigen *	Isotype	K _a (10 ⁴ /Ms)	K _d (10 ⁻⁵ /s)	K _D (10 ⁻¹⁰ M)	Rmax (RU)Δ
Histone 2A	IgG3	1.5±0.3	21.9±1.4	143.2±22.0	68.1
	IgG1	0.5±0.1	17.4±1.2	360.2±18.9§	57.6
	IgG2b	1.1±0.0	80.5±5.7	739.1±40.2§	11.5
	IgG2a	9.0±1.4	61.4±4.5	68.4±11.6§	134
Histone 2B	IgG3	1.2±0.2	12.0±1.0	102.7±15.4	112.2
	IgG1	0.3±0.0	7.6±1.2	249.1±27.1§	74.6
	IgG2b	1.5±0.9	69.9±6.4	452.7±35.5§	70.5
	IgG2a	7.0±0.6	40.1±3.8	57.4±9.8§	162.4

* Histone was immobilized to chip at a concentration of 5 nM.

ΔMaximum value of response unit at the range of 0 to 200 nM of Ab.

§Compared to IgG3, p < 0.05.

Note: The K_D values were calculated according to the equation $KD = Kd/K_a$. The K_a and K_d values of isotype-specific binding to histones were calculated using the results obtained at different Ab concentrations (0, 20, 40, 80, 120, and 200 nM).

Table 2

The wavelength and energy changes of tryptophan fluorescence

Ag	Ab	Wavelength change (nm)	Energy change (kJ/mol)
dsDNA	IgG3	3.04±0.07	-3.115 ^Δ
	IgG1	0.17±0.05 [*]	-0.120
	IgG2b	1.21±0.22 [*]	-1.318 ^Δ
	IgG2a	0.14±0.06 [*]	-0.144
ssDNA	IgG3	6.20±0.12	-6.201 ^Δ
	IgG1	3.33±0.07 [*]	3.520 ^Δ
	IgG2b	1.35±0.14 [*]	1.427 ^Δ
	IgG2a	3.06±0.10 [*]	-3.161 ^Δ
Histone 2A	IgG3	3.64±0.50	3.538 ^Δ
	IgG1	1.07±0.11 [*]	1.146 ^Δ
	IgG2b	2.42±0.07	2.587 ^Δ
	IgG2a	0.85±0.02 [*]	0.884
Histone 2B	IgG3	1.12±0.23	1.121 ^Δ
	IgG1	5.24±0.47 [*]	-5.508 ^Δ
	IgG2b	3.82±0.24 [*]	4.008 ^Δ
	IgG2a	2.41±0.49	2.530 ^Δ

Note: The changes in energy were calculated using the de Broglie equations, $E = hc/\lambda$. E is energy in Joules (J), h is the Planck constant ($6.626069 \times 10^{-34} \text{ J}\cdot\text{s}$), c is the speed of light ($2.997924 \times 10^8 \text{ m/s}$) and λ is either the initial or final emission wavelength measured.

^{*} $p < 0.05$, compared to the PL9-11 IgG3 isotype.

^Δ $p < 0.05$, compared to the unbound state.