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Human Complement Receptor 2 (CR2/CD21) as a Receptor for DNA: Implications for its Roles in the Immune Response and the Pathogenesis of Systemic Lupus Erythematosus (SLE)

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Abstract

Human CR2 is a B cell membrane glycoprotein that plays a central role in autoimmunity. Systemic lupus erythematosus (SLE) patients show reduced CR2 levels, and complete deficiency of CR2 and CR1 promotes the development of anti-DNA antibodies in mouse models of SLE. Here we show that multiple forms of DNA, including bacterial, viral and mammalian DNA, bind to human CR2 with moderately high affinity. Surface plasmon resonance studies showed that methylated DNA bound with high affinity with CR2 at a maximal K_D of 6 nM. DNA was bound to the first two domains of CR2 and this binding was blocked by using a specific inhibitory anti-CR2 mAb. DNA immunization in *Cr2*$^{-/-}$ mice revealed a specific defect in immune responses to bacterial DNA. CR2 can act as a receptor for DNA in the absence of complement C3 fixation to this ligand. These results suggest that CR2 plays a role in the recognition of foreign DNA during host-immune responses. This recognition function of CR2 may be a mechanism that influences the development of autoimmunity to DNA in SLE.

Keywords

Autoimmunity; B Lymphocyte; Complement Receptor 2; DNA Receptor; Protein-DNA interactions; Surface Plasmon Resonance

1. Introduction

Human complement receptor type 2 (CR2/CD21) is a multi-functional receptor expressed primarily on B cells and follicular dendritic cells (FDC), although a subset of peripheral and

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Conflict of interest
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thymic T cells also express CR2 [reviewed in (Ahearn and Fearon, 1989; Holers, 2001)]. CR2 has four well-characterized classes of ligands, including complement C3 fragments iC3b, C3dg and C3d (Iida et al., 1983), each of which are covalently attached to target antigens (Ags), as well as the gp350/220 viral coat protein of the Epstein-Barr virus (Fingeroth et al., 1984), the immunoregulatory protein CD23 (Aubry et al., 1992), and interferon-alpha (IFN-α) (Asokan et al., 2006).

On B lymphocytes CR2 is thought to act primarily as a co-receptor for B cell antigen receptor (BCR)-mediated signal transduction. On B cell lines or primary B lymphocytes, co-ligation of CR2 with surface IgM, using either mAbs (Carter et al., 1988), model immune complexes (Thornton et al., 1996), covalently linked complexes of antigen with C3d ligand (Dempsey et al., 1996) or biotin-conjugated C3dg complexed with biotinylated anti-IgM (Boackle et al., 2001) results in enhanced intracellular calcium release, proliferation, upregulation of B7-1 and B7-2 co-stimulatory molecules on B lymphocytes, and/or activation of tyrosine kinases and MAP kinases. This enhancing activity is due to the association of CR2 with CD19 and CD81 in a BCR specific signal transduction complex (Cambier et al., 1994; Matsumoto et al., 1991). On FDC, CR2 acts to trap C3 ligand-bound antigens, presumably to hold these on the surface for interactions with germinal center B cells (Molina et al., 1990).

Ligation of CR2 alone on B cells by C3d or gp350, in the absence of BCR co-ligation, results in several additional phenotypes, including the induction of homotypic adhesion (Nichols et al., 1998), NF-κB activation (Sugano et al., 1997), IL-6 generation (D'Addario et al., 2001), antigen uptake and presentation to T cells (Boackle et al., 1997; Carel et al., 1990) and actin polymerization (Melamed et al., 1994).

Support for a critical role of CR2 in the immune response has been provided by results in mice that are deficient in CR2 [as well as mouse complement receptor type 1 (CR1)]. In mice, CR2 is encoded along with the larger receptor CR1 by the *Cr2* gene, which produces both proteins through alternative splicing of a common mRNA (Kurtz et al., 1990; Molina et al., 1990). *Cr2*−/− mice demonstrate marked decreases in antigen-specific, T-dependent and T-independent humoral immune responses (Ahearn et al., 1996; Molina et al., 1996) that is due to a lack of receptor on both B cells and FDCs (Fang et al., 1998), as well as defects in B cell memory (Wu et al., 2000).

Relevant to the current study, in addition to this defect in response to foreign antigens, recent studies have also suggested that CR2 plays a key role in regulating tolerance to certain nuclear self antigens such as single and double strand DNA, chromatin and histones. That is because autoimmune-prone mice either lacking expression of CR2 and CR1 through gene targeting or expressing a dysfunctional CR2 molecule display enhanced autoreactivity to this class of autoantigens (Boackle et al., 2001; Prodeus et al., 1998). The molecular explanation for this phenotype is not understood. One proposal suggests that enhanced autoimmunity to nuclear antigens in the relative or complete absence of CR2 and/or CR1 function may be due to the requirement for complement and CR2/CR1 in Ag capture in the bone marrow (Prodeus et al., 1998), and another that it may be due to ineffective deletion of autoreactive B cells by C3/C4-bound antigens (Boackle et al., 2001; Carroll, 2004; Prodeus et al., 1998). However, despite the uncertainties regarding the exact role of CR2 in maintaining tolerance to DNA, histones and chromatin, markedly lower expression of human CR2 in patients with systemic lupus erythematosus (SLE) (Levy et al., 1992) and mouse models of SLE before the onset of detectable humoral autoimmunity (Takahashi et al., 1997) have suggested that this function of CR2 may be pathophysiologically important in the development of this autoimmune disease. The effect is not major, however, as MRL/

lpr mice lacking CR2/CR1 expression do not show markedly altered changes in autoantibody levels (Boackle et al., 2004).

It has been known for several decades that patients with SLE and murine models exhibit anti-DNA and anti-chromatin Abs and that these autoantibodies are likely to be pathogenic in disease (Hahn et al., 1997). Recently, however, the complexity of DNA structural variants such as non-methylated CpG-rich sequences that are directly immunostimulatory molecules for multiple lymphoid cell types (Krieg, 2002), the potential for different recognition mechanisms for types of DNA derived from mammalian as well as bacterial sources (Pisetsky, 1996), the ability of non-CpG rich DNA to activate B cells using unknown receptors (Vollmer et al., 2002), and an important role for bacterial and other pathogen DNA as an immunogen recognized during immune responses to pathogens (Wloch et al., 1997) have been increasingly recognized. In addition, both free DNA and DNA within immune complexes in serum are readily detectable using contemporary techniques such as PicoGreen labeling detection (Bjorkman et al., 2003).

Studies to identify and characterize DNA and chromatin receptors were originally fraught with technical difficulties and poor reproducibility. However, recently substantial advances have been made in demonstrating that Toll like receptor 9 (TLR9) can function to mediate biologic effects of CpG rich immunostimulatory DNA (Sabroe et al., 2003), and that TLR9 is also important in the amplification of rheumatoid factor expressing B cells to immune complexes containing IgG and nucleosomes (Leadbetter et al., 2002). On B cells, CpG immunostimulatory DNA may also be able to interact with the BCR to impart an additional activation signal (Liang et al., 2001). However, the roles of these receptors in lupus pathogenesis as well as in either tolerogenic or immunogenic B and T cell responses to self or foreign DNA and DNA-containing chromatin are not yet clearly defined.

In these studies we report that CR2 can serve as an apparent receptor for DNA and chromatin in the absence of C3 fixation to these ligands. CR2 exhibits readily detectable interactions with multiple forms of DNA using surface plasmon resonance and ELISA approaches. The highest affinity interaction of CR2 with this class of ligands appears to be with methylated DNA. This result suggests that the DNA binding function evolved as a means of recognizing bacterial DNA in the absence of the requirement for complement fixation. Consistent with this hypothesis, CR2-deficient mice demonstrate a deficiency in the humoral immune response following immunization with bacterial DNA that is much greater than mice deficient in the CR2 ligand C3. Based on these results, we propose that a deficiency of CR2 may also contribute to the pathogenesis of SLE through altered interactions with and/or clearance of DNA.

2. Materials and Methods

2.1 Human CR2 SCR1-4-BCCP expression vector

Human SCR1-4-BCCP was prepared as described in the previously published method (Asokan et al., 2006). Briefly, Human CR2 SCR1-4 was amplified by PCR from a previously utilized CR2-encoding expression vector (Molina et al., 1995) using the primers 5′-CCG GGC CAG CCG GGC CAT TTC TTG TGG CTC TCC TCC GCC-3′ and 5′-GAA TGC GGC CGC ACT ACT AAA AAT TTC TTC ACA TAC TGG CAT TTT GG-3′ that also generate *Sfi* and *Not* 1 restriction endonuclease enzyme sites at the 5′ and 3′ ends, respectively. A clone containing amino acids 70-156 of the biotin carboxyl carrier protein (BCCP) was generously provided by Dr. John Lambris (Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA), as was a clone encoding the biotin holoenzyme synthetase protein Bir A, which catalyzes the incorporation of biotin into BCCP (Sarrias et al., 2001). A Bir A enzyme kit was also purchased from

Avidity (Denver, CO). Sequences encoding CR2 SCR1-4 -BCCP were then cloned into the pSecTag2/Hygromycin resistant vector (Invitrogen, Inc., Carlsbad CA) such that soluble SCR1-4 would be expressed with carboxy-terminal BCCP. Before transfection, cDNA underwent nucleotide sequence analysis in the University of Colorado Denver Cancer Center DNA sequencing core to make sure the presence of the anticipated DNA sequence in the construct.

2.2 Transfection, expression and purification of human CR2 SCR1-4-BCCP

Human CR2 SCR1-4-BCCP transfection, expression and purification were performed as described in the previously published method (Asokan et al., 2006). Briefly the cDNA encoding the recombinant protein SCR1-4-BCCP was stably expressed in the NSO and HEK 293 mammalian cell lines using electroporation and Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA), respectively. For NSO cell line expression, the CR2 SCR1-4-BCCP cDNA was linearized and then electroporated at 100 microfarads/300 V in 0.5 ml of a PBS solution containing 50 μg herring sperm DNA and 6 μg SCR1-4-BCCP cDNA. Cells were then serially diluted in media under selection with 800 μg/ml hygromycin. For HEK 293 cell expression, cells were recovered by 0.1% trypsin treatment and release from a flat bottom flask, counted and then seeded at 2.5×10^5 in 500 μl growth medium containing no serum and no antibiotics. For transfection of the CR2 SCR1-4-BCCP cDNA, 1 μg of linearized plasmid and 3 μl Lipofectamine 2000 in 50 μl Opti-MEM reduced serum medium was mixed and incubated at 37° C for 30 min, followed by incubation in tissue culture wells for 24–48 hrs. Following this, cells were diluted serially and grown in the presence of 800 μg/ml hygromycin. In both expression systems, after 2–3 weeks individual clones were recovered and screened for CR2 protein expression by ELISA method. The secreted protein obtained from both of these cell line supernatants was concentrated and stored at −70 °C until sufficient volume was ready for purification.

Soluble human CR2 SCR1-4-BCCP protein was purified as described in the previously published method (Asokan et al., 2006). The stored −70 °C supernatant was thawed at RT, filtered, concentrated and dialyzed in to 50 mM PBS overnight at 4° C. Dialyzed supernatant was loaded on a previously equilibrated HB-5-Sepharose 4B anti-CR2 monoclonal antibody (which specifically recognizes an epitope in CR2 SCR 3-4) affinity column at 4° C. Following washing of unbound protein, the bound protein was eluted using a buffer previously described (Weis et al., 1986) to maintain CR2 activity that contains 0.2 M acetic acid, 150 mM NaCl, 1 mM PMSF, 1 μM leupeptin and 1 μM pepstatin, pH 2.5. The eluate was immediately neutralized with 1.0 M Tris buffer, pH 7.4. The eluted protein was pooled, concentrated and dialyzed in 50 mM PBS overnight at 4° C. All CR2 proteins purified through this method were then finally passed through a Protein G-Sepharose column in order remove a trace amount of anti-CR2 monoclonal antibody HB-5 that leaches from the column during this purification method.

2.3 Human CR2 SCR1-15 expression and purification

Human CR2 SCR 1-15 produced in baculoviral Sf9 cells was produced as described in the method (Guthridge et al., 2001b). Briefly, human CR2 SCR 1-15 was expressed in Sf9 insect cells as a recombinant soluble protein in the baculovirus expression system. The cDNA coding for the first 15 SCR domains plus the native human Kozac consensus sequence and signal peptide as a soluble protein was cloned into the pVL1393 baculoviral expression vector (Pharmingen, San Diego, CA). After infection of the insect cells with the recombinant baculovirus, spinner cultures were grown for 5 days at 27°C. The supernatants were removed, filtered and concentrated, and the concentrate was dialyzed overnight into 50 mM PBS at 4°C. To purify CR2 SCR1-15, the dialyzed supernatant was loaded on a previously equilibrated HB-5 Sepharose 4B monoclonal antibody column, and protein was

purified as noted above for CR2 SCR1-4. Positive fractions were pooled and concentrated. The protein size and purity was assured by both non-reducing and reducing SDS-PAGE analysis using NuPAGE 10% Bis-Tris gels and MES NuPAGE running buffer (Invitrogen).

2.4 Human C3d expression and purification

Human C3d was produced and purified as previously described method (Guthridge et al., 2001b). Briefly, human C3d was expressed by transfection of the pET15b-C3d plasmid into BL21 pLysS Codon Plus *Escherichia coli* (Stratagene). The culture were grown and induced with 25 mM isopropyl β-D- thiogalactoside (IPTG) at 28° C and shaken overnight. The bacterial pellet was harvested in DEAE starting buffer (20 mM Tris, pH 7.1) plus Complete EDTA-free protease inhibitor tablets (Roche-Boehringer Mannheim), and lysis was performed using four freeze thaw cycles. DNase (4,000 U) and RNase (50 μl of 0.5 mg/ml stock) were added, and the lysate was clarified by sonication and centrifugation at 8, 0000 × g. The C3d supernatant obtained was purified using a DEAE HiPrep column, followed by a MonoQ HR5 column in 20 mM Tris, pH 7.1 and a MonoS HR5 column in 50 mM MES, pH 7.0, where in all three cases C3d was eluted using a linear salt gradient up to 1 M NaCl. The protein purity was confirmed using SDS-PAGE.

2.5 EBV gp350 expression and purification

EBV gp350 protein was produced and purified as described (Hedrick et al., 1994). Briefly, a 70 kDa fragment of the Epstein-Barr virus gp350/220 coat protein was cloned into the pVI-Bac transfer vector (Pharmingen, San Diego, CA). This contained a melittin signal sequence and a recombinant protein with a C-terminal polyhistidine tag. This fragment of gp350/220 was produced by infecting Sf9 insect cells with the gp350/220-packaged baculovirus particles. The cultures were grown and the culture supernatant was processed as described above for CR2 SCR 1-15. The 70 kDa fragment of gp350/220 was then purified using a Con-A Sepharose column. The filtered and concentrated supernatant was loaded on a previously equilibrated column. The unbound protein was washed off with 20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl. The bound protein was eluted with increasing concentration of alpha-D-methylmannoside or alpha-D-methylglucoside. The eluted protein was concentrated, dialyzed into PBS and stored at −20°C. Protein purity was assessed by SDS-PAGE.

2.6 15, 25, 100bp adenoviral E1a DNA preparation and purification

15 (5′-TTC TAT GCC AAA CCT-3′), 25 (5′-TTC TAT GCC AAA CCT TGT GCC GGA G-3′) and 100 bp (nucleotide sequences not shown) adenoviral E1a DNA complement strand obtained from Operon Biotechnologies Inc, Huntsville, AL was mixed in equal molar of each DNA in annealing buffer containing 10 mM HEPES + 50 mM NaCl, pH 7.5 in 1 ml Volume. Then the DNA was annealed at 95°C water bath and left overnight to cool down at cold room temperature. The annealed dsDNA fragment was separated from ssDNA by purification using S75 gel filtration column chromatography with 10 mM HEPES + 50 mM NaCl, pH 7.5 at 4°C. The positive dsDNA fraction collected was concentrated using centricon, aliquoted and stored at 4°C until further use.

2.7 Structurally modified DNA preparation and purification

16bp Scrambled DNA (ATG CAT GCT GAC TAC G), 50% CpG rich DNA (TTC GAT CGC ACG CCC G) and 50% methylated DNA (TTC GAT CGC ACG CCC G) was annealed and purified as mentioned above. The positive fraction collected was concentrated using centricon filtered, aliquoted and stored at 4°C until further use.

2.8 PGEM plasmid DNA preparation

PGEM-3 plasmid DNA was prepared as cited (Emlen et al 1990). Briefly, PGEM-3 plasmid (Promega, Madisson, WI) was grown in *E. coli* and plasmid DNA was isolated by cell lysis followed by centrifugation over cesium chloride gradient. Plasmid DNA was cut a single site with endonuclease Sma-1 (Bethesda Research Labs, Gaithersburg, MD) yielding a linear, blunt-end dsDNA molecule of 2867 bp analysis of this DNA on 0.8% agarose gels demonstrated a single homogenous band. After ethanol precipitation, DNA was brought to a concentration of 1 mg/ml in 10 mM HEPES + 50 mM NaCl, pH 7.4, aliquoted and stored at 4°C until further use.

2.9 Calf thymus chromatin preparation

Calf thymus chromatin was prepared as described (Portanova et al., 1987). Briefly, chromatin was obtained from Calf thymus nuclei and was depleted of H1 and non histone proteins with 0.6 M NaCl. H1 depleted chromatin was diluted to 1 mg DNA/ml with 0.1 mM Na_2 EDTA, pH 7.2 and was digested with an equal volume of TPCK-treated trypsin (Worthington Biochemical Corp., Cochranville, PA) at 2 μg/ml in 0.02 M Sodium phosphate buffer, pH7.2, containing 0.28 M NaCl. Digestion proceeds for 90 min at 37°C and was terminated by the addition of 50 mM PMSF (Calbiochem-Behring Corp., La Jolla, CA) to a final concentration of 1mM, aliquoted and stored at 4°C until further use.

All other DNA including *E. coli* HMW DNA and *Micrococcus luteus* DNA were purchased from Sigma (St. Louis, MO).

2.10 Bir A enzyme expression and purification

Bir A enzyme expression and purification was performed as described (Asokan et al., 2006). Briefly, the biotin holoenzyme synthetase protein, Bir A, catalyzes the incorporation of biotin into BCCP. This protein was expressed in *E. coli* using a clone that was provided by Dr. John Lambris and purified (Sarrias et al., 2001). Following growth at 37°C in YT medium containing 50 μg/ml ampicillin, 0.25 mM IPTG was added to induce protein expression. The expressed protein in the cell lysate was purified by Ni-NTA affinity column. Briefly, the cell lysate was dialysed in 10 mM Tris, 300 mM NaCl, 10 mM imidazole pH 8.0 overnight at 4°C. The dialyzed supernatant was filtered and incubated with 5 ml of Ni-NTA resin for 60 min at 4°C and then packed into a column. The unbound protein was washed with 10 mM Tris with 20 mM imidazole pH 8.0. The bound protein was eluted with increasing concentrations of imidazole (0.01–0.25 M) in 0.02 M PBS (pH 7.5) containing 0.5 M NaCl. The eluted protein was dialyzed against 50 mM Tris, 200 mM KCl and 5% glycerol (pH 7.5) at 4°C and stored at −70°C.

2.11 Site specific biotinylation of human CR2 SR1-4-BCCP using the bir A enzyme

Site specific biotinylation was performed as described in the method (Sarrias et al., 2001). To accomplish this, CR2 SCR1-4-BCCP protein was dialyzed against 40 mM Tris containing 5.5 mM $MgCl_2$ and 100 mM KCL (pH 8.0) overnight at 4°C. Following this, 30 μg SCR1-4-BCCP protein was incubated with 365 μg purified Bir A protein, 24 μg d-biotin and 20 mM ATP for 1 hr at 37°C. Excess biotin was removed by a desalting pD-10 column followed by extensive dialysis against PBS. Protein was stored at −70°C in aliquots until use.

2.12 SDS-PAGE and Western blot analysis of recombinant proteins

Protein size and purity was determined using non-reducing SDS-PAGE and Western blot analysis as described in the method (Asokan et al., 2006). Briefly, purified CR2 SCR1-4-BCCP was electrophoresed on a NUPAGE Novex 10% Bis-TRIS gel with MOPS NUPAGE

as the running buffer. Proteins were then transferred to a nitrocellulose membrane and incubated sequentially with anti-CR2 mAb HB-5 and peroxidase-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). In another blot the same proteins were transferred to a nitrocellulose membrane to evaluate for biotinylation efficiency. In both experiments, non-specific binding was blocked with PBS 0.05% Tween 20 containing 10% milk. The incorporation of biotin was assessed by reactivity with peroxidase-conjugated SA (BD PharMingen, San Diego, CA) at 1 μg/ml. Proteins were detected using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). In addition, N-terminal amino acid sequence analysis was performed for the CR2 SCR1-4-BCCP protein to confirm the correct protein identity. To accomplish this, a 12% denaturing minigel was prepared and run with SDS-PAGE running buffer containing 100 mM reduced glutathione at 37°C and stored overnight at 4°C before loading the sample. Next, CR2 SCR1-4-BCCP protein was electrophoresed in running buffer prepared by substituting glutathione with 2-ME. Bands were transferred to PVDF membrane, and the membrane was stained with 0.1% Coomassie Blue in 50% methanol for 5 min. Following slight de-staining, the transferred protein bands were excised with a razor blade, and N-terminal sequence analysis was then performed in the Protein Sequencing Core (National Jewish Medical and Research Center, Denver, CO).

Similarly, the protein size and purity of human CR2 SCR1-15, human C3d and EBV-gp350 was assessed by non-reducing SDS-PAGE. The protein was electrophoresed on a NUPAGE Novex 10% Bis-TRIS gel with MOPS NUPAGE as the running buffer for 60 min and then stained with 0.05% coomassie brilliant blue staining.

2.13 Measurement by ELISA of the interaction of CR2 with its DNA ligands

Dose-dependent binding of CR2 to its DNA ligands was studied using the ELISA method in which 2 μg/ml DNA from various sources or control BSA were immobilized on 96 well ELISA plates (Costar) in 150 mM NaCl, 15 mM sodium citrate buffer pH 8.0 or reacti-bind DNA coating solution (Pierce, Rockford, IL) overnight at 4°C. Plates were then washed and blocked with PBS Tween 20 containing 1% BSA. Serial dilutions of CR2 SCR1-15 were then added to the wells in PBS containing 50 mM NaCl, pH 7.4 and allowed to incubate for 1 hr binding at 37°C. After washing, CR2 SCR1-15 bound to DNA was detected using anti-CR2 mAb HB-5, which is specific for SCR3-4, followed by anti-goat mouse IgG (Jackson ImmunoResearch Laboratories) at 1:1000. Color was developed using ABTS, and the OD was measured at an absorbance of 405 nm.

2.14 Cross-competition analysis using CR2 protein ligands C3d and gp350

To determine the relationships between the DNA binding site and other CR2-ligand interaction sites, 2 μg/ml *Micrococcus luteus* DNA was coated on a 96 well ELISA plates (Costar) in 150 mM NaCl, 15 mM sodium citrate buffer pH 8.0 or reacti-bind DNA coating solution (Pierce, Rockford, IL) overnight at 4°C. Plates were then washed and blocked with PBS Tween 20 containing 1% BSA. Molar fold excess of BSA, C3d, gp350 and DNA from *Micrococcus luteus* were made on a diluter plate. CR2 SCR1-15 at 2 μg/ml was then added to the serially diluted wells, as well as to control wells with buffer alone, and incubated for 1 hr at RT. These samples were then added to DNA bound ELISA plates and incubated for 1 hr. Following washing, the amount of CR2 SCR1-15 bound to the wells was measured using anti-CR2 mAb HB-5 as above.

2.15 Measurement by surface plasmon resonance of the interaction of CR2 with protein and DNA ligands

CR2 binding to its ligands human C3d and gp350 and various DNA ligands was studied using BIAcore 3000 (BIAcore, Piscataway, NJ) with streptavidin (SA)-coupled chip. All

experiments were performed in 10 mM HEPES, 50 mM NaCl, 1 mg/ml carboxymethyl-dextran, and 0.05% surfactant P-20, pH 7.4 at 25°C. The chip regeneration buffer used 1 M NaCl in place of 50 mM NaCl. For kinetic analysis biotinylated CR2 SCR1-4-BCCP (1250 RU) were immobilized on a SA chip. Immobilization was performed on either flow cell (FC) 2 or FC4, and FC1 and FC3 were used as control cells. Binding was measured at 30 μl/min to avoid mass transfer effects. At this flow the initial on-rate was maximal. Flow was allowed for several seconds to establish baseline, and then various concentrations of analyte were injected. The association was allowed for 120 s and the dissociation of the complex was monitored for 120 s for protein ligands. For DNA ligands 180 s, 360s was allowed for both association and dissociation phase to allow optimal binding. BIA evaluation 3.1 (BIAcore) software was used to analyze the binding data, using global fitting where best fit was indicated by a low residual and chi^2 values <10. This analysis yields an association rate k_a, a dissociation rate k_d and a dissociation constant K_D for protein-protein and protein-DNA interactions.

2.16 Inhibition of CR2-ligand interaction by mAbs recognizing the first two SCRs of CR2

In order to determine whether the first two SCR domains were directly involved in CR2 binding to 25bp E1a DNA, PGEM plasmid DNA, *E. coli* HMW DNA and *Micrococcus luteus* DNA, CR2 SCR1-15 was pre-incubated for 1 hr at 37°C with IgG1 anti-CR2 mAbs (171, 1048, 994, 629) directed to the SCR1-2 domain (Guthridge et al., 2001b) or a control IgG1 anti-human factor B mAb (gift of Dr. Joshua Thurman). Preincubated CR2 SCR1-15 samples were then added for 1 hr at 37°C to an ELISA plate that had been pre-coated with 25bp E1a DNA, PGEM plasmid DNA, *E. coli* HMW DNA and *Micrococcus luteus* DNA in 150 mM NaCl, 15 mM sodium citrate buffer pH 8.0 or reacti-bind DNA coating solution (Pierce, Rockford, IL) overnight at 4°C. The amount of CR2 SCR1-15 bound to the wells was detected using the anti-CR2 mAb HB-5 followed by goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a 1:1000 dilution. Color was developed using ABTS and OD measured at an absorbance of 405 nm. Percent inhibition was calculated by:

$$1-[(\text{Average Experimental OD}-\text{Background OD})/(\text{Maximum Average OD}-\text{Background OD})]\times 100.$$

2.17 Anti-DNA antibody production against bacterial DNA

Micrococcus luteus DNA (Sigma, St. Louis, MO) 1 mg/ml was dissolved in 850 μl PBS. 75 μg mBSA (Sigma, St. Louis, MO) dissolved in 1 ml PBS separately. 150 μl of mBSA was added to the 850 μl DNA in PBS and vortex and mixed thoroughly for 15 min. Then, 1 ml of Imject Complete Freund's Adjuvant (Pierce, Rockford, IL) was added and mixed thoroughly. Finally two top needle and syringes was used to mix well before going for injection. A 100 μl mixture containing 50 μg *Micrococcus luteus* DNA with mBSA was injected intra peritonially to 5 mice from each group of C57BL/6 *Cr2+/+*, *Cr2−/−* and *C3−/−* mice. Mice were then re-immunized at the same concentration on day 14 and 28. Mice were bled by tail vein every 7 days and the serum analyzed for levels of anti-DNA antibody production.

To measure anti-DNA antibodies, ELISA titration was performed against *Micrococcus luteus* DNA using sera from C57BL/6 *Cr2+/+*, *Cr2−/− and C3−/−* mice. 5 μg/ml *Micrococcus luteus* DNA was coated on the plate in 150 mM NaCl, 15 mM sodium citrate buffer pH 8.0 or reacti-bind DNA coating solution (Pierce, Rockford, IL) overnight at 4°C. Plates were then washed and blocked with PBS Tween 20 containing 1% BSA and incubated for 1 hr. Sera from C57BL/6 *Cr2+/+*, *Cr2−/− and C3−/−* mice were serially diluted with PBS, added to the plate and incubated for 1 hr. After washing, the level of bound anti-DNA antibody was detected using peroxidase conjugated goat-anti mouse IgG

(Sigma, St. Louis, MO) diluted 1:1000 in PBS and added to the plate and incubated for 1 hr. Color was developed with the substrate solution 3, 3′, 5, 5′-teteramethylbenzidine (Sigma, St. Louis, MO) and the reaction was stopped at 10 min by adding 1 mM H_2 SO_4. Color development was measured at an optical density of 450.

2.18 Protein and DNA determination

The absorption coefficient for each protein calculated at A_{280} was used to determine protein concentrations. For DNA $A_{260\text{-}280}$ was measured and used to calculate concentrations.

2.19 Statistical analysis

Analyses were performed using two tail Student's *t* test.

3.0 Results

3.1 Generation and characterization of recombinant proteins and DNA for binding studies

The ligand binding site within the amino-terminal two short consensus repeats (SCR) of CR2 has previously been reported for C3d, gp350, CD23 and IFN-α. To study these CR2-DNA interactions in comparison to protein ligand C3d and gp350, we utilized one previously described form of soluble recombinant CR2 containing SCRs 1-15, which interacts with C3d and gp350 (Asokan et al., 2006; Guthridge et al., 2001a). In addition, for this current study we used a new form of soluble recombinant CR2 SCR 1-4 with BCCP, a biotinylation site, at the carboxyterminus region. The addition of the SCR3-4 domain allowed us to detect binding of this protein to ligands with the non-ligand-blocking mAb HB-5, which reacts with the SCR3-4 domain. In addition, the incorporation of a biotinylation site at the carboxy-terminus allowed us to reproducibly orient the ligand binding domain of CR2 to a SA chip, as previously described (Sarrias et al., 2001).

We utilized the mammalian cells NSO and HEK 293 to express CR2 SCR1-4-BCCP, and proteins were purified using mAb HB-5-Sepharose 4B affinity column as described (Asokan et al., 2006). The same protein were used for this study.

The ligands C3d were expressed in *E. coli* and purified as described (Guthridge et al., 2001a; Szakonyi et al., 2001). EBV-gp350 was also expressed and purified using an established method (Hedrick et al., 1994).

For CR2-DNA interactions studies, we prepared various forms of DNA fragments originating from viral, bacterial and mammalian sources such as 15, 25, 100bp adenoviral E1a DNA, PGEM plasmid DNA, *Micrococus luteu*s DNA (Sigma, St Lious, MO), *E. coli* HMW DNA (Sigma, St Louis, MO), and calf thymus chromatin, respectively. In addition to that, we have utilized structurally modified DNA fragments in order to determine whether bacterial CpG DNA as well as methylated DNA. 16bp scrambled DNA, 50% CpG rich DNA and 50% methylated DNA showed any distinguished binding with CR2. All reagents were prepared as described in the Material and Methods section.

3.2 Dose-dependent binding of CR2 to DNA ligands

Figure 1A demonstrates the results of experiments in which DNA from viral, bacteria and mammalian origin, or BSA as a control, is first bound to an ELISA plate. Subsequently, decreasing doses of CR2 SCR1-15 are added, followed by washing and detection of bound CR2 by anti-CR2 mAb HB-5. These results demonstrate a remarkable consistency in which specific dose-dependent interactions are readily detected with each of the DNA-containing ligands tested.

3.3 Cross-competition analysis

To determine the relationships between the protein-ligand binding sites and DNA-ligand binding sites, the relative ability of CR2 SCR1-15 to interact with plate bound *Micrococcus luteus* DNA was determined using cross-competition ELISA. Figure 1B demonstrates the results of this analysis and reveals a rank order of competition of gp350>*Micrococcus luteus* DNA>C3d. This likely reflects the relationships between ligand binding sites, although we cannot rule out a contribution to the results by the relative differences in affinities. Nevertheless, the results are consistent with the presence of closely related binding sites for each ligand.

3.4 Kinetic analysis of the interaction of CR2 SCR1-4 with protein and DNA-ligand by surface plasmon resonance

In this study, we have examined the interaction of CR2 with protein and DNA-ligands by surface plasmon resonance using a Biacore 3000 to compare the binding kinetics of protein and DNA ligands for surface-attached CR2 SCR1-4. In these experiments, carboxy-terminal biotin-tagged CR2 SCR1-4-BCCP on a SA chip was used and the ligands human C3d and EBV-gp350 were included as solution phase analytes to compare with DNA. Binding activity was measured and calculated were the association and dissociation rates as well as the K_D for each of the ligands (Figure 2A–E, Table 1).

In the first experiments, we examined the interaction of 15, 25 and 100bp adenoviral E1a DNA as a solution phase analyte to the immobilized CR2 SCR1-4-BCCP on a SA chip. We found that the binding reaction was dose-dependent but not saturable at the highest tested concentration of 125 nM. Among the different bp E1a DNA tested, 15bp E1a DNA showed a much higher affinity than the 25 and 100bp E1a DNA tested with a k_D 245, 358 and 769 nM respectively. These values are comparable to C3d and gp350, which demonstrated dose-dependent binding that was saturable at the highest tested concentration of 125 nM and with a k_D 278 and 417 nM, respectively (Figure 2A–E, Table 1).

In the second set of experiment (Figure 3A–C) we used bacterial DNA including PGEM plasmid DNA (histogram not shown), *E.coli* HMW DNA and *Micrococcus luteus* DNA and mammalian DNA calf thymus chromatin. Among the bacterial DNA tested, DNA from *Micrococcus luteus* showed highest affinity with a k_D of 10 nM. Mammalian DNA calf thymus chromatin showed higher affinity with a k_D of 50 nM.

We used BIA evaluation software version 3.1 to evaluate whether these ligand-receptor pairs exhibited simple 1:1 binding or whether more than one binding site could be detected. The data analysis revealed that the binding reaction of the experimental curve (colored line) does not fit well to a simple 1:1 Langmuir binding model but rather fits to a two-site binding model (solid lines). These analyses suggest the presence of one high affinity binding site and a second much lower (2–3 logs or greater difference) affinity binding site in the CR2 SCR1-4 domain. For this interaction, we report only the higher affinity binding site K_{D1} values (Table 1). Chi^2 values are the goodness of fit parameter describing how precisely the experimental curve fits to the proposed binding model. The chi^2 values range from 1.1–10.0 (Table I) and show that a two-site binding model fits well to the experimental data.

3.5 Kinetic analysis of the role of structurally modified DNA interaction with CR2 SCR1-4 by surface plasmon resonance

In this experiment we tested whether 16bp scrambled DNA, 50% CpG DNA and 50% methylated DNA showed any different affinity, is that can be comparable to un-scrambled 15bp E1a DNA (Figure 4A–C; Table 2). In this experiment, we examined the interaction of above structurally modified DNA as solution phase analyte to the immobilized CR2

SCR1-4-BCCP on a SA chip. We found that the binding reaction was dose-dependent but not saturable at the highest tested concentration of 125 nM. Among the structurally modified DNA tested 50% methylated DNA showed higher affinity with a K_D of 6 nM compared 16bp scrambled and 50% CpG DNA with a K_D of 266 nM and 509 nM, respectively.

We used BIA evaluation software version 3.1 to evaluate whether these ligand-receptor pairs exhibited simple 1:1 binding or whether more than one binding site could be detected. The data analysis revealed that the binding reaction of the experimental curve (colored line) does not fit well to a simple 1:1 Langmuir binding model but rather fits to a two-site binding model (solid lines). These analyses suggest the presence of one high affinity binding site and a second much lower (2–3 logs or greater difference) affinity binding site in the CR2 SCR1-4 domain. For this interaction, we report only the higher affinity binding site K_{D1} values (Table 2). Chi^2 values are representing the goodness of fit parameter describing how precisely the experimental curve fits to the proposed binding model. The Chi^2 values range from 1.0–6.9 (Table 2) and show that a two-site binding model fits well to the experimental data.

3.6 Mapping of DNA- receptor ligand interactions using informative anti-CR2 mAbs

Previous studies using inhibitory mAbs as well as cross-competition of ligands have demonstrated that the binding sites for C3d, gp350 and CD23 overlap within the SCR1-2 domain (Guthridge et al., 2001b; Martin et al., 1991). In the next set of experiments, we determined whether a set of anti-CR2 mAbs generated to and reactive with the SCR1-2 domain that we had previously created (Guthridge et al., 2001b) would inhibit the binding of DNA to CR2. As shown in Figure 5, a very similar pattern of inhibition is found when comparing the relative ability of mAbs to block DNA binding to CR2 as found when comparing them to C3d (mAb 171>1048>994>629) as reported previously (Asokan et al., 2006; Guthridge et al., 2001b). These results suggest that each ligand binds to a closely related site on CR2 SCR1-2.

3.7 In vivo immune response

Immunization experiments were performed to see whether CR2 plays a role in anti-DNA antibody production following bacterial *Micrococcus luteus* dsDNA immunization. We utilized C57BL/6 *Cr2+/+*, *Cr2−/−* and *C3−/−* mice. *Cr2−/−* mice re-immunized at day 14 showed reduced anti-DNA antibody production compared to *Cr2+/+*, $p < 0.001$; and *C3−/−* mice, $p < 0.01$; indicating impaired anti-DNA antibody production (Figure 6). *C3−/−* mice did not show any change as compared to *Cr2+/+* mice, which suggested that a CR2 dependent immune response to DNA is not dependent upon C3 fixation.

4.0 Discussion

Previous studies of the interaction of CR2 with C3d and gp350 have identified a positively charged patch on CR2 that comprises a significant portion of the ligand binding site (Hannan et al., 2005; Kovacs et al., 2009; Kovacs et al., 2010; Szakonyi et al., 2006; Young et al., 2007), while the C3d binding site for CR2 is negatively charged (Morikis and Lambris, 2004). Similarly, it is well known that positively charged Arg residues play critical roles in the affinity of monoclonal anti-DNA antibodies for this target antigen. These observations led us to the hypothesis that the development of anti-DNA autoantibodies and the responses to DNA-containing B cell activators could be regulated by CR2 itself serving as a receptor for DNA.

Several initial approaches were taken to test the hypothesis that human CR2 interacts with DNA. First, we utilized full length CR2 to measure interaction with DNA ligands. We utilized an ELISA method where the ligands are placed on the well and then soluble

recombinant CR2 SCR1-15 is tested for binding identically as we have done before for protein ligands (Asokan et al., 2006; Guthridge et al., 2001a) (note that SCR1-15 binding is detected by anti-CR2 mAb that does not disrupt ligand binding). Each of these types of DNA-containing ligands interacts with CR2. Recently another group has shown the binding of complement receptor 1 (CR1, CD35) to CpG-DNA by flowcytometry analysis (Lepse et al., 2011). It is known that the binding sites for two CR2 ligands, C3d and gp350, while closely related and both found in the SCR1-2 domain, are not identical (Hannan et al., 2005; Young et al., 2007; Kovacs et al., 2009 and 2010). To determine whether the DNA binding site is preferentially related to one or the other site, we have performed cross-competition analyses. DNA binding to CR2 is not substantially inhibited by C3d, but is by gp350, suggesting that *Micrococcus luteus* DNA masks the EBV-gp350 binding site preferentially

We have also utilized additional investigative methods including surface plasmon resonance (Biacore), a technique, which has been used to study the CR2-C3d and CR2-gp350/220 interaction (Asokan et al., 2006; Guthridge et al., 2001a; Sarrias et al., 2001). Herein we have used carboxy-terminal biotin-tagged CR2 SCR1-4-BCCP on a streptavidin chip and solution phase analytes as shown. The protein ligand C3d and gp350 showed similar affinity as reported earlier (Asokan et al., 2006). The E1a DNA fragments 15bp showed higher affinity than the 25bp and 100bp. Comparing protein ligand and E1a DNA, both showed comparable affinities. Analysis of this interaction with C3d and gp350/220 as well as three dsDNA fragments from the adenoviral E1a gene revealed the sequence of the 25bp fragment is TTC TAT GCC AAA CCT TGT GCC GGA G, which does not contain the canonical CpG sequence and is equally balanced between purines and pyrimidines. We also evaluated binding of DNA to cells expressing CR2; however, high non-specific background binding of DNA to non-expressing cells did not allow the reproducible detection of CR2-dependent binding.

We then utilized four additional forms of DNA to determine whether this property of CR2 was widely shared or specific to the original sequence. PGEM plasmid DNA (histogram not shown) used for anti-dsDNA assays (Emlen et al., 1990), *E. coli* and *Micrococcus luteus* DNA each interact with CR2. Furthermore, calf thymus chromatin interacts with CR2. Of interest, even though DNA exhibits multiple repetitive sites for recognition, at the density of CR2 on the chip the best fit model based on these data is a 1:1 interaction with a primary high affinity site and also a markedly lower affinity site on CR2.

Furthermore, we have utilized structurally modified DNA fragments 16bp scrambled DNA, 50% CpG DNA and 50% methylated DNA resulted nice dose-dependent binding among which methylated DNA gave a maximum K_D of 6 nM.

In the present study we have utilized low salt buffer (1/3 PBS, 50 mM NaCl, pH 7.4) for these interaction studies. At high salt buffer (1/3 PBS, 150 mM NaCl, pH 7.4) DNA did not show high affinity binding (data not shown). Similarly it has been reported that at 50 mM NaCl CR2-C3d complex occurs but at 137 mM NaCl it disappears (Li et al., 2008). The loss of CR2–C3d interaction in 137 mM NaCl may be explained by masking of electrostatic interaction that mediate complex formation in 50 mM NaCl. Recently the co-crystal structure of CR2 SCR 1-2-C3d complex by (van den Elsen and Isenman, 2011) reported that the interaction interface markedly differed and was more consistent with biochemical data than the previous co-crystal structure published (Szakonyi et al., 2001). However, the CR2 interface in the new structure still contains the positively charged patch (van den Elsen and Isenman, 2011).

There is a possibility that cell surface CR2 can act as a DNA binding receptor in order to bind, internalize and degrade the exogenous circulating DNA. The immune stimulatory

property of DNA has been implicated in the pathogenesis of systemic lupus erythematosus (Lamphier et al., 2006). It has been shown that unmethylated CG dinuleotides are responsible for most immunostimulatory properties of microbial DNA or synthetic CpG ODN (Krieg et al., 1995). We have also shown that CR2 interacts with the 25 bp E1a fragments using gel retardation analyses (Gerda Szakonyi and Xiaojiang Chen, unpublished data). In addition to that CR2 SCR1-15 and 100bp E1a DNA interaction was achieved using 1/3 PBS by analytical ultracentrifugation analysis suggesting 1:1 interaction (data not shown). These additional technical analyses strongly reveal that DNA binds to CR2. Furthermore, anti-CR2 inhibitory antibody raised against the first 2 SCR domain revealed the first two SCR directly involved in binding all of the DNA fragments tested as reported for CD23, C3d and gp 350 (Asokan et al., 2006; Guthridge et al., 2001b; Martin et al., 1991).

In vivo experiments with *Micrococcus luteus* DNA immunization of *Cr2+/+*, *Cr2−/−* and *C3−/−* revealed *Cr2−/− m*ice re-immunized at day 14 showed reduced anti-DNA antibody production compared to *Cr2+/+*, $p < 0.001$ and *C3−/−* mice which are statistically significant at $p < 0.01$ indicated impaired anti-DNA anti body production. *C3−/−* mice did not show any change as compared to *Cr2+/+* mice indicated that CR2 is a high affinity receptor for DNA in the absence of C3 fixation.

Conclusions

Human CR2 can act as a receptor for DNA in the absence of complement C3 fixation to this ligand. These results suggest that CR2 plays a role in the recognition of foreign DNA during host-immune responses. Alterations of this recognition function of CR2 may be an underlying mechanism that helps to regulate self tolerance and development of autoimmunity to DNA in SLE.

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Highlights

- Human CR2 can act as a receptor for DNA in the absence of complement C3 fixation to this ligand.
- CR2 plays a role in the recognition of foreign DNA during host-immune responses.
- Alterations of this recognition function of CR2 may be an underlying mechanism that helps to regulate self tolerance and development of autoimmunity to DNA in SLE.

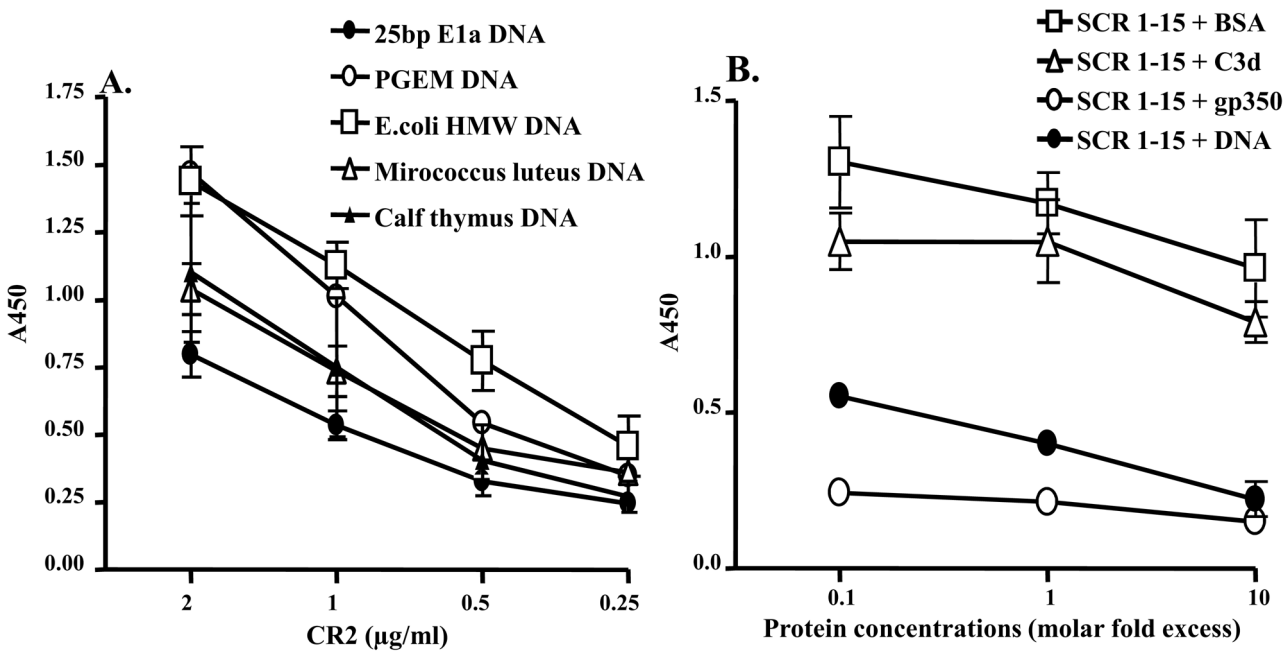

Figure 1.

Figure 1A. Dose-dependent binding measured by ELISA of CR2 SCR1-15 to its plate-bound DNA ligands from viral, bacterial and mammalian origin. Binding was measured using non-blocking anti-CR2 mAb HB-5 that interacts with CR2 SCR3-4. Mean ± SD of three experiments is shown. Figure 1B. Cross-competition ELISA measuring relative ability of C3d, gp350 and DNA to block CR2 SCR1-15 interactions with plate-bound *Micrococcus luteus* DNA. Mean ± SD of three experiments is shown.

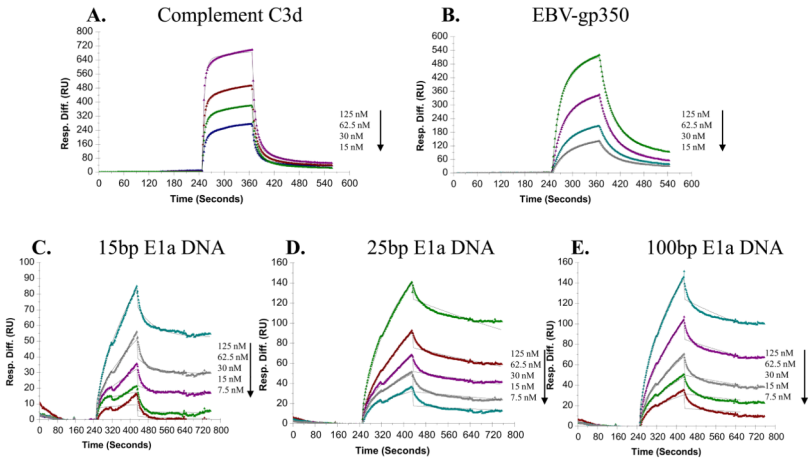

Figure 2.

Surface plasmon resonance sensograms demonstrating the association and dissociation of protein ligands C3d (A) and EBVgp350 (B) as well as viral DNA fragments, 15 bp E1a DNA (C), 25 bp E1a DNA (D) and 100bp E1a DNA (E) with immobilized biotinylated CR2 SCR1-4-BCCP. Nanomolar concentrations of injected analyte are indicated at the right hand side of each sensogram. Solid lines are the result of global fitting analysis, and the colored lines are the normalized experimental sensogarm kinetic data.

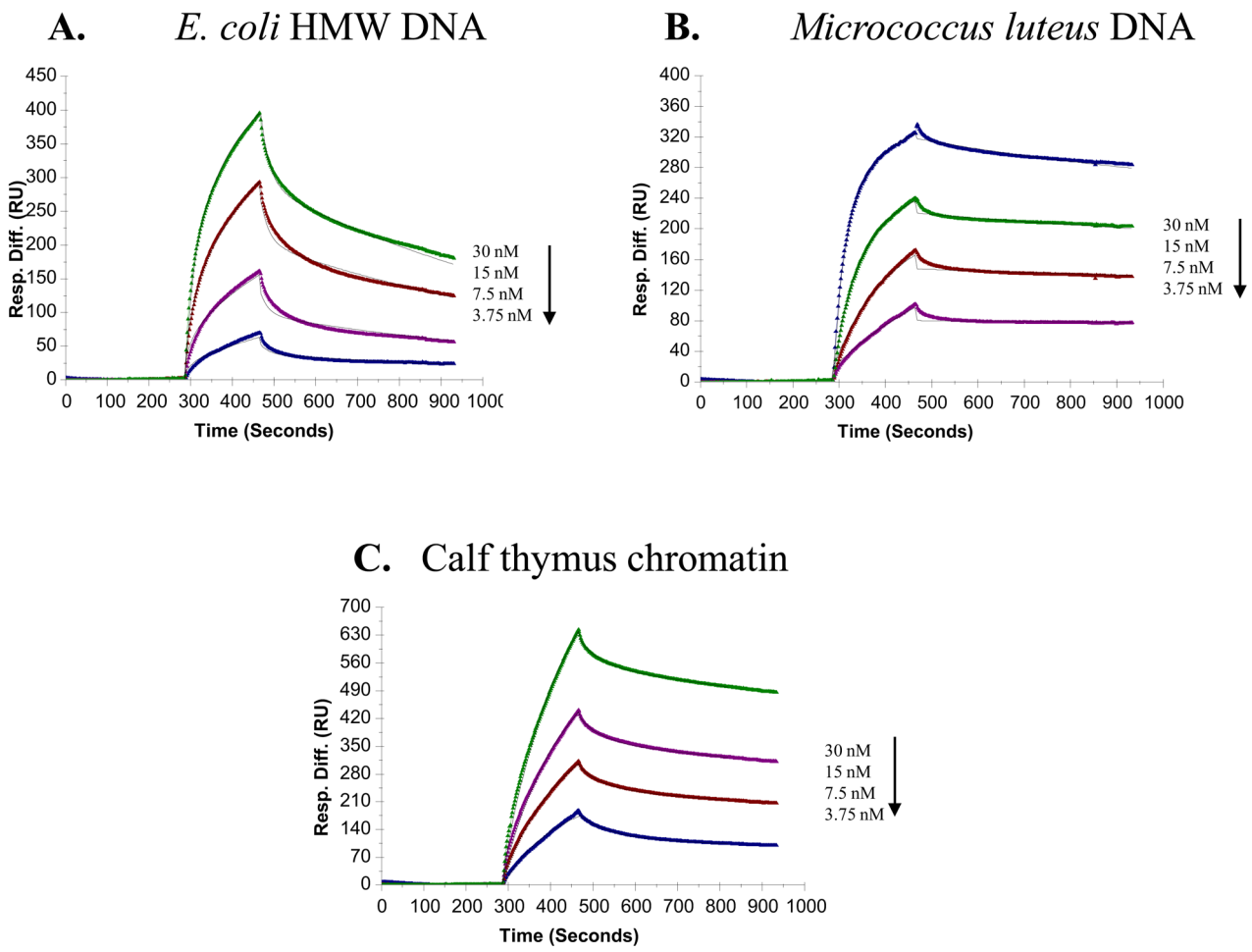

Figure 3.

Surface plasmon resonance sensograms demonstrating the association and dissociation of bacterial DNA ligands E. coli HMW DNA (A), *Micrococcus luteus* DNA (B) and Calf thymus chromatin (C) with immobilized biotinylated CR2 SCR1-4-BCCP. Nanomolar concentrations of injected analyte are indicated at the right hand side of each sensogram. Solid lines are the result of global fitting analysis, and the colored lines are the normalized experimental sensogarm kinetic data.

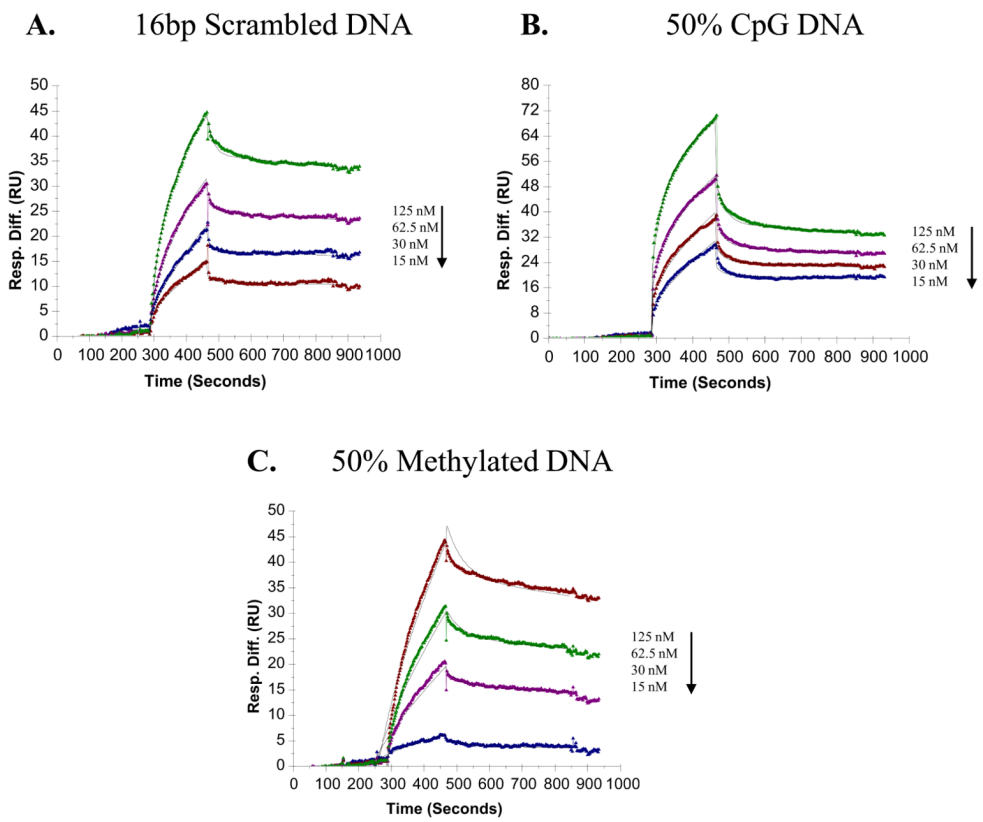

Figure 4.

Surface plasmon resonance sensogram demonstrating the association and dissociation of structurally modified DNA including 16bp scrambled DNA (A) 50% CpG DNA (B) and 50% methylated DNA (C) with immobilized biotinylated CR2 SCR1-4-BCCP. Nanomolar concentrations of injected analyte are indicated at the right hand side of each sensogram. Solid lines are the result of global fitting analysis, and the colored lines are the normalized experimental sensogarm kinetic data.

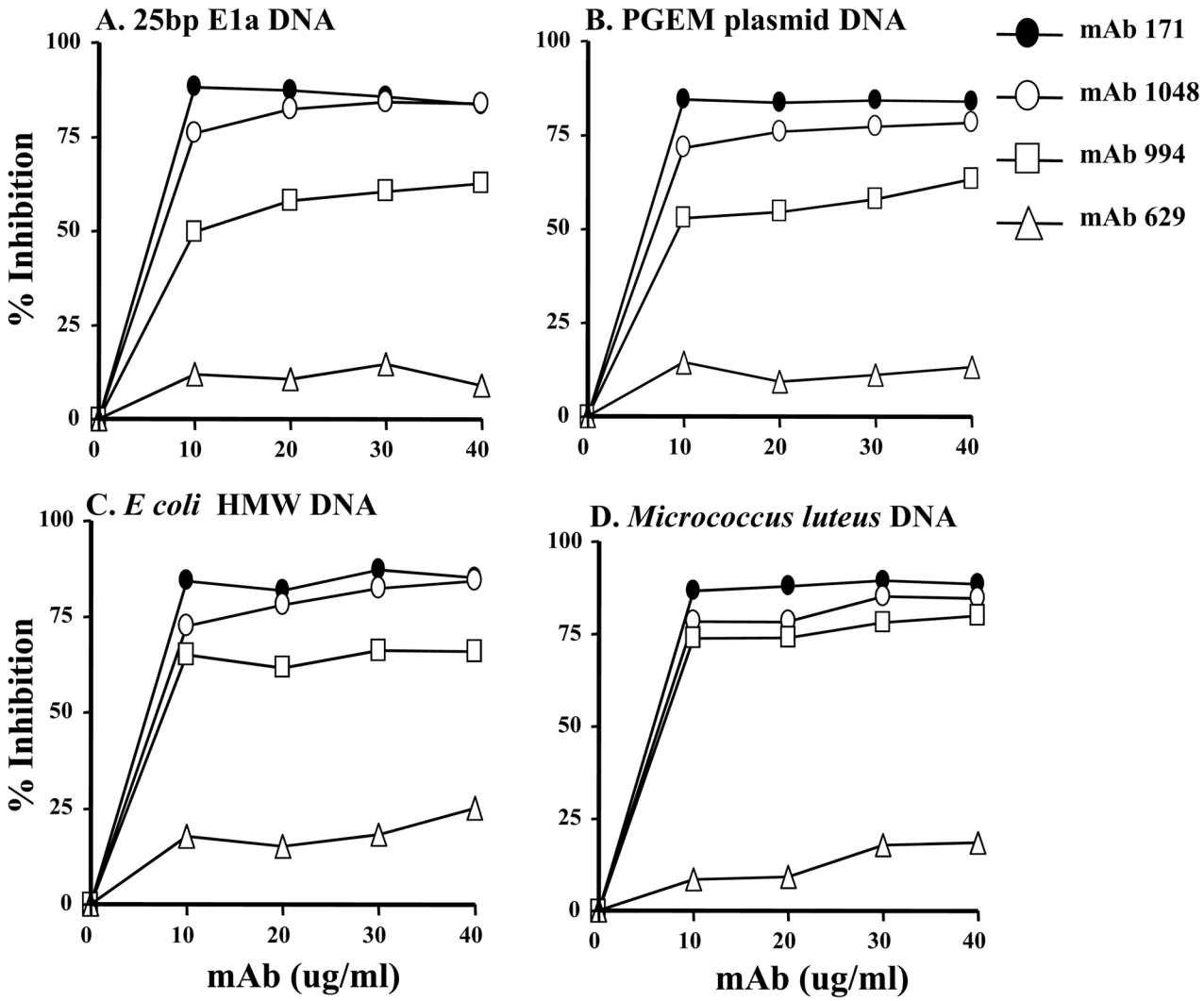

Figure 5.

Competition ELISA measuring relative ability of anti-CR2 mAbs to block soluble CR2 SCR1-15 binding to plate-bound DNA ligand, 25bp E1a DNA (A), PGEM plasmid DNA (B) E.coli HMW DNA (C) and *Micrococcus luteus* DNA (D). Binding was measured using anti-CR2 mAbs HB-5 that will bind to CR2 SCR3-4 and not compete with any of these four mAbs tested. Results demonstrate that mAbs 171, 1048, 994 and 629 are inhibitory in the same relative rank order of each ligand. Mean of three representative experiments is shown.

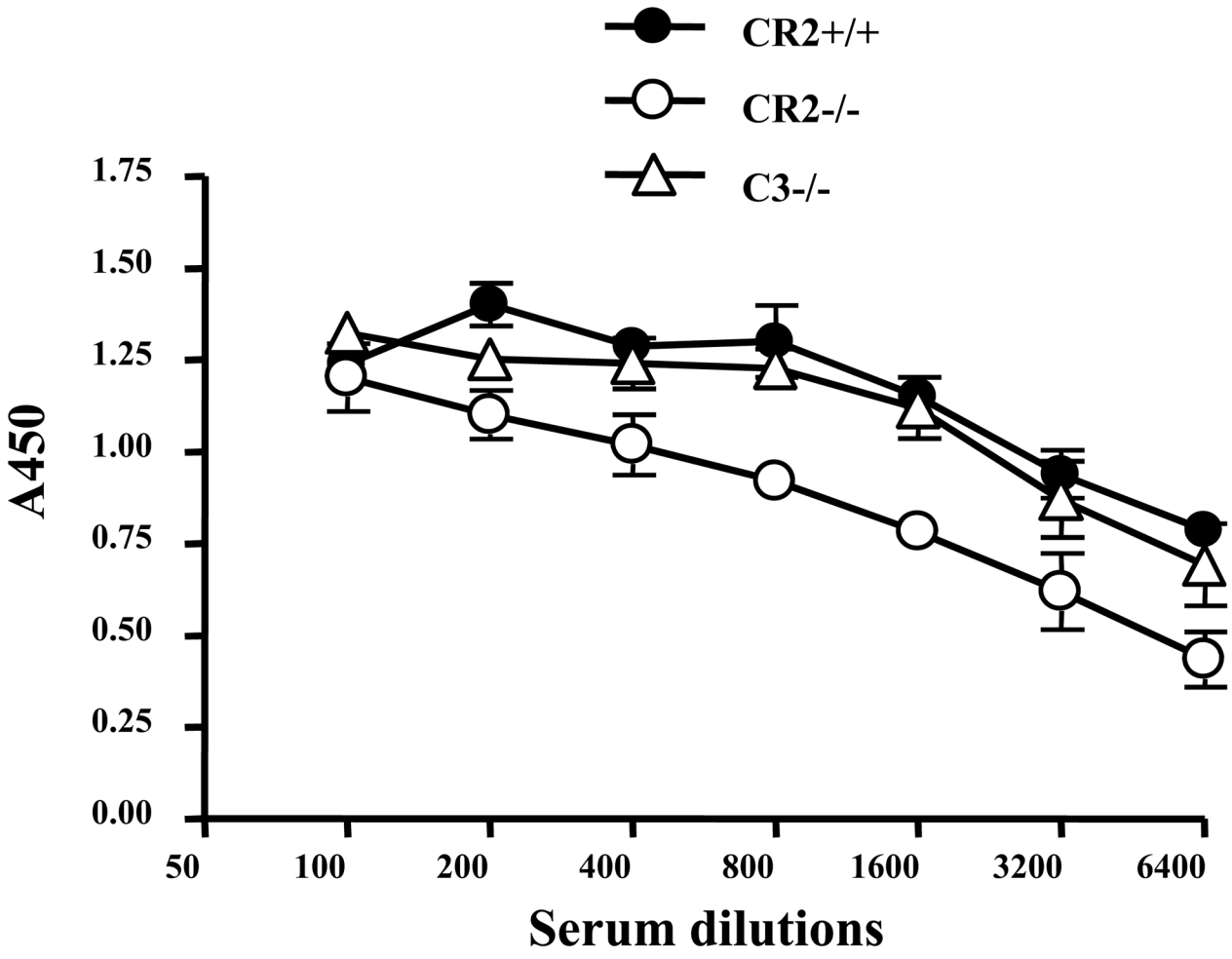

Figure 6.

Titration of anti-DNA antibody production against *Micrococcus luteus* dsDNA immunized sera from C57BL/6 *Cr2+/+, Cr2−/−* and *C3−/−* mice. *Micrococcus luteus* dsDNA at 5 ug/ml was coated overnight at 4°C. Sera from immunized C57BL/6 *Cr2+/+, Cr2−/−* and *C3−/−* were serially diluted with PBS and loaded on the plate. Level of anti-DNA bound was probed with peroxidase-conjugated goat anti-mouse IgG. Mean ± SD for three experiments is shown. *Cr2−/−* mice re-immunized at day 14 showed reduced anti-DNA antibody production compared to *Cr2+/+*, $p < 0.001$; and *C3−/−* mice, $p < 0.01$; indicating impaired anti-DNA antibody production.

Table 1

Kinetic values for the interaction of CR2 with its protein and DNA ligands

Ligand	Analyte[a]	k_{d1}(1/s)/k_{a1}(1/M s)[b]	K_{D1} [c]	χ^{2}[d]
	Protein ligands			
CR2	C3d	$6.4\times10^{-2}/2.3\times10^{5}$	0.278 μM	3.1
CR2	EBV-gp350	$3.3\times10^{-2}/7.9\times10^{4}$	0.417 μM	2.8
	Viral DNA PCR fragments			
CR2	15bp E1a DNA	$1.3\times10^{-2}/5.3\times10^{4}$	0.245 μM	5.0
CR2	25bp E1a DNA	$1.9\times10^{-2}/5.3\times10^{4}$	0.358 μM	1.9
CR2	100bp E1a DNA	$2.0\times10^{-2}/2.6\times10^{4}$	0.769 μM	1.1
	Bacterial DNA			
CR2	PGEM plasmid DNA	$6.7\times10^{-2}/7.5\times10^{5}$	0.089 μM	0.19
CR2	*E. coli* HMW DNA	$2.2\times10^{-2}/2.3\times10^{5}$	0.095 μM	5.0
CR2	*Micrococcus luteus* DNA	$2.7\times10^{-3}/2.7\times10^{5}$	0.010 μM	4.0
	Mammalian DNA			
CR2	Calf thymus chromatin	$9.2\times10^{-3}/1.8\times10^{5}$	0.05 μM	10.0

[a] Experiments performed at 50 mM NaCl (Average values represent from 3–4 determinations).

[b] Ka, on rate; kd, off rate.

[c] Equilibrium dissociation constant (kd/ka).

[d] Chi-squared goodness-of-fit parameter.

Table 2

Kinetic values for the interaction of CR2 with its structurally modified DNA ligands

Ligand	Analyte[a]	kd1(1/s)/ka1(1/M s)[b]	K_{D1}[c]	χ^2[d]
	Highly characterized DNA			
CR2	16bp Scrambled DNA	$4.0 \times 10^{-2}/1.5 \times 10^5$	0.266 μM	1.0
CR2	50% CpG DNA	$5.6 \times 10^{-2}/1.1 \times 10^5$	0.509 μM	1.8
CR2	50% Methylated DNA	$2.0 \times 10^{-3}/4.1 \times 10^5$	0.006 μM	3.5

[a] Experiments performed at 50 mM NaCl (Average values represent from 3–4 determinations).

[b] Ka, on rate; kd, off rate.

[c] Equilibrium dissociation constant (kd/ka).

[d] Chi-squared goodness-of-fit parameter.