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The human complement receptor type 2 (CR2)/CR1 fusion protein TT32, a novel targeted inhibitor of the classical and alternative pathway C3 convertases, prevents arthritis in active immunization and passive transfer mouse models☆

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ABSTRACT

Complement activation in human diseases is characterized by the local covalent deposition of the long-lived C3 fragments iC3b/C3dg/C3d. Previously, TT30, a complement alternative pathway (AP)-selective inhibitor, was designed as a fusion protein linking the first four short consensus repeats (SCRs) of human complement receptor type 2 (CR2) with the first five SCRs of human factor H (fH). TT30 acts by utilizing CR2 SCR1–4 to bind the initially formed iC3b/C3dg/C3d fragments and delivering surface-targeted inhibition of AP C3 and C5 convertases through fH SCR 1-5. In order to combine classical (CP) and lectin (LP) pathway inhibitory abilities employing CR2-mediated targeting, TT32 was developed. TT32 is a CR2-CR1 fusion protein using the first ten SCRs of CR1, chosen because they contain both C3 and C5 convertase inhibitory activity through utilization of decay-acceleration and cofactor activity for both AP and CP. In Wieslab assays, TT32 showed potent inhibition of the CP and AP with IC_{50} of 11 and 46 nM, respectively. The TT32 inhibitory activity is partially blocked with a molar excess of a competing anti-CR2 mAb, thus demonstrating the importance of the CR2 targeting. TT32 was studied in the type II (CII) collagen-induced arthritis (CIA), an active immunization model, and the CII antibody-induced arthritis (CAIA) passive transfer model. In CIA, injection of 2.0 mg TT32 at day 21 and 28 post disease induction, but not untargeted CR1 alone, resulted in a 51.5% decrease in clinical disease activity (CDA). In CAIA, treatment with TT32 resulted in a 47.4% decrease in CDA. Therefore, a complement inhibitor that targets both the AP and CP/LP C3/C5 convertases was shown to limit complement-mediated tissue damage and inflammation in disease models in which all three complement activation pathways are implicated.

1. Introduction

The complement system is a crucial part of the innate immune response and plays a central role in helping the host resist infection by employing a variety of mechanisms – anaphylatoxin generation, opsonization, lysis of pathogens as well as enhancing the adaptive immune response (Ricklin and Lambris, 2013). The complement system is composed of more than 50 proteins, and three different activation mechanisms have been identified – the classical, lectin and alternative pathways. The complement classical pathway (CP) is triggered

Abbreviations: CP, classical pathway; AP, alternative pathway; LP, lectin pathway; MAC or C5b-9, membrane attack complex; CAIA, collagen antibody-induced arthritis; CIA, collagen-induced arthritis; CII, type II collagen; DAS, disease activity score; AJM, all joint mean; IC, immune complex; NBF, neutral buffered formalin; RA, rheumatoid arthritis; SCRs, short consensus repeats; RCA, regulators of complement activity; sCR1, soluble complement receptor1; CR1, complement receptor 1; CR2, complement receptor 2

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predominantly by antigen-antibody complexes, while the complement lectin pathway (LP) is activated by the binding of pattern recognition molecules such as mannose-binding lectin (MBL), ficolins and collectins to oligosaccharide and other structures on microorganisms and self-tissues. Activation of both the CP and the LP leads to the generation of the enzyme complex C4b2a, known as the CP C3 convertase, which cleaves C3 into C3a and C3b. In contrast, activation of the alternative pathway (AP) is independent of the binding of specific recognition molecules. Rather, constitutive low level activation of the AP occurs through the spontaneous hydrolysis of an internal thioester bond in C3 (tickover process), leading to formation of C3(H_2O) (Bexborn et al., 2008). The altered structure of C3(H_2O) enables binding of factor B (fB), and the resulting C3(H_2O)B complex is cleaved by factor D (fD), thus generating the fluid phase C3 convertase C3(H_2O)Bb. Similar to the CP/LP C3 convertase, the fluid phase AP C3 convertase cleaves C3 into the anaphylatoxin C3a and the larger C3b that can covalently bind to near-by surfaces of cells. When surface bound C3b combines with fB, factor D cleaves the C3bB complex to form a surface bound AP C3 convertase (C3bBb) (Harrison, 2018), leading to further generation of C3b and C3a. Properdin is a positive regulator of complement activation that stabilizes the AP convertases (C3bBb). Properdin promotes the association of C3b to FB and can also provide the initial platform for the assembly of AP convertase (Hourcade, 2006). Finally, the positive feedback mechanism, the amplification loop, can quickly multiply the amount of surface deposited C3b. Additional C3 molecules interact with the AP/CP/LP C3 convertases to form the C5 convertases C3bBbC3b and C4b2aC3b, wherein the K_M for C5 is dramatically lowered (Rawal and Pangburn, 2001a, b). C5 convertases cleave C5 into the anaphylatoxin C5a as well as the larger fragment C5b, which then initiates the assembly of C6, C7, C8 and polymeric C9 molecules, ultimately forming a 100 Å wide pore in the membrane, called the membrane attack complex (MAC, C5b6789$_n$), which can then lead to cell lysis (Bayly-Jones et al., 2017).

Given the highly reactive nature of the complement system and its ability to quickly generate anaphylatoxins and damage cells, the host needs to tightly regulate the system on the cell surface in order to avoid injury to self-tissues. Indeed, many of the host's cells express several regulators of complement activity (RCA) that can function on cell surfaces or in the fluid phase. Examples of RCA molecules include factor H, CR1 (CD35), decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), membrane inhibitor of reactive lysis (MIRL, CD59) and C4b-binding protein (C4bp)(Noris and Remuzzi, 2013). These RCAs can inactivate the C3/C5 convertases by dissociation (decay-accelerating activity) and/or by promotion of factor I mediated proteolysis of C3b (cofactor activity), the latter process generating the C3b fragments iC3b and C3dg, which is subsequently cleaved to C3d by another protease mechanism (Stoermer and Morrison, 2011). C3b and its proteolytic products can interact with different receptors (CRIg, CR1, CR3, CR4, CR2), triggering different physiological responses (Wiesmann et al., 2006). The binding of antigen-bound iC3b, C3dg or C3d to complement receptor 2 (CR2, CD21) expressed on B cells, in a process that brings together the CR2-associated CD19 molecular signal-enhancing function with the B cell receptor, is of particular importance since it contributes to increased production of antibodies, thus linking innate and adaptive immunity (Carroll, 1998; Carroll and Prodeus, 1998; Sarrias et al., 2001).

CR2 is a 145 kDa type I transmembrane glycoprotein containing 15 or 16 short consensus repeats (SCRs) followed by a 28 amino acid transmembrane domain and a 34 amino acid intra-cytoplasmic domain. Each SCR is composed of approximately 60 amino acids and connected by a 4–8 amino acid linker peptide (Fujisaku et al., 1989; Weis et al., 1988). In mice, CR2 is encoded by the single *Cr1* gene that generates two receptors from a common mRNA by alternative splicing (Kurtz et al., 1990; Molina et al., 1990). In humans, two different genes on chromosome 1 encode two different receptors, CR1 and CR2. CR2 is not only expressed on B cells but also on T cells, follicular dendritic cells, mast cells, and basophils (Carroll, 1998). *Cr2* $^{-/-}$ mice have defects in B cell memory and antibody generation (Chen et al., 2000).

The role of CR1/CR2 in mouse models of arthritis is somewhat controversial. In one study it has also been shown that CR2/CR1 deficiency protects mice from collagen-induced arthritis (CIA) (Kuhn et al., 2008), while in another study CR1/CR2 deficiency enhanced CIA in female mice but not in male mice under low-antigen conditions (Nilsson et al., 2009). While reduced B-cell expression of CR1/CR2 has been reported in humans with rheumatoid arthritis (RA) in one study (Prokopec et al., 2010), the expression of CR2 on peripheral B cells of RA patients was found to be equivalent to that of healthy subjects in another study. However, CR2 expression was absent on synovial B cells from RA patients in that study (Illges et al., 2000). These studies indicate that CR1/CR2 may play a role in regulating autoimmunity.

Dysregulation of complement activity has been implicated in several additional pathological conditions, e.g., age-related macular degeneration (AMD), dense deposit disease (DDD), thrombotic microangiopathies (TMA), cold agglutinin disease (CAD), paroxysmal nocturnal hemoglobinuria (PNH), systemic lupus erythematosus (SLE), IgA nephropathy as well as ischemia and reperfusion injury (I/R) (Ricklin and Lambris, 2013). Given that complement activation via the AP, CP and LP or a combination of these has been implicated in the aforementioned diseases, we decided to design and test an inhibitor of all three activation pathways that was also targeted to areas of complement activation.

Previous studies have demonstrated that proteins consisting of the C3 fragment binding domains of murine CR2 fused to complement regulators (CR2-Crry and CR2-fH) were efficacious in mouse models of CIA and collagen antibody-induced arthritis (CAIA) (Banda et al., 2009; Song et al., 2007), and also demonstrated targeting to the joints in a tissue deposited C3 fragment-dependent manner, along with a durable clinical benefit, but in the presence of a very short circulating half-life (Song et al., 2007). To advance this targeting method, we designed a human fusion protein, denoted TT32, which contains the C3-fragment binding domains of human CR2 (CD21) fused to the AP and CP inhibitory domains of human CR1. CR1 consists of four long homologous repeats (LHR A–D), each containing seven SCR repeats. It has been shown that the first three SCRs contain the complement regulatory activity of a given LHR (Makrides et al., 1992). Moreover, the SCRs 8–10 of LHR B (site 2) are almost identical to the SCRs 15–17 of LHR C and possess an identical inhibitory profile that is different from the SCRs 1–3 of LHR A (site 1). Site 1 is a weak binder of C3b and an intermediate binder of C4b and exhibits weak cofactor activity and high decay acceleration activity. The potency profile of site 2 is almost opposite – C3b is bound with high affinity, cofactor activity is high and decay acceleration activity is low (Krych-Goldberg and Atkinson, 2001). Therefore, the first ten SCR domains of CR1 contain all of the key modalities required for pan-complement inhibition, acting as cofactors for irreversible proteolytic cleavage of C3b or C4b as well as decay-accelerators for AP and CP C3 and C5 convertases.

TT32 is designed to localize AP/CP inhibitory activity to cell surfaces that are under complement attack. This is different from TT30 (CR2-fH), a targeted inhibitor of the AP convertases only (Fridkis-Hareli et al., 2011). Here we compare the in vitro potency of TT32 to that of TT30 in complement activation assays. We also examined the in vivo effect of TT32 on the CIA and CAIA mouse models of RA. In addition, a positive control was included through the use of an anti-mouse C5 mAb that has previously demonstrated efficacy in this model (Wang et al., 1995).

2. Materials and methods

2.1. Design, expression, and purification of human TT32

A plasmid containing full length TT32 cDNA was synthesized by DNA2.0 (Menlo Park, CA) based on sequences of CR2 and CR1 which

are listed in Genbank. The sequence encoding SCRs 1–4 of human CR2 (NP_001006659.1, amino acid residues 21–275) was fused 5′ to the sequence encoding SCRs 1–10 of human CR1 (NM_000573.3, amino acid residues 42–687). Human embryonic kidney 293 F cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) and the tissue culture supernatant was loaded onto a capto MMC column, followed by hydrophobic interaction chromatography (either butyl sepharose or MEP hypercel resins), and lastly, performing cation exchange on SP sepharose. The fractions from the cation exchange step were pooled and evaluated for complement targeting activity. Size and purity of the final pool was evaluated using SDS PAGE. One μg was loaded onto a 4–20% Tris-glycine gel and stained with Coomassie blue to visualize the band of TT32. Gels, stains and standards were from Invitrogen.

2.2. Determination of the binding affinity of TT32 to C3d and C3b by biacore®

The binding of purified TT32 to C3d and C3b was evaluated by using surface plasmon resonance (SPR) (Biacore, GE). Purified TT32 was coupled to a CM5 chip using standard amine coupling and varying concentrations of C3b or C3d were flowed over the surface. The surface was regenerated with 20 mM HCL, 0.01% P20. KD was calculated using steady state equilibrium analysis. Principles of SPR are reviewed in (Drescher et al., 2018).

2.3. Wieslab complement system alternative/classical (AP/CP) ELISA assays

Serum samples from all mice for all functional assays were prepared for this study according to the methods described by Lachman (Lachmann, 2010). The Wieslab™ Complement System Alternative and Classical Pathway Enzyme ELISA kits (Euro-Diagnostica, Malmo, Sweden) assess the degree of AP or CP activity by measuring the amount of active C5b-9 formation using an immunoassay format. During incubation of serum in the wells, the AP/CP is activated in a buffer containing pathway-specific metal ions and inhibitors, and C5b-9 is deposited on the coated wells. The wells of the microtiter strips are pre-coated with pathway-specific activators (endotoxin-LPS for AP and IgM for CP). Bound C5b9 is detected with alkaline phosphatase-conjugated mouse monoclonal antibody mAb aE11that recognizes the C9 neoantigen formed during MAC assembly. The amount of C9 neoantigen generated is directly proportional to the functional activity of the AP or CP. These kits were used to assess the effect of TT32, TT30, sCR1 (R&D Systems), CR1 1–10 (Alexion) and anti-CR2 mAbs (clones 171, 629 (Alexion) and 1048 (Green Mountain)) on each pathway, according to manufacturers' instructions. TT32 and other compounds were diluted in normal complement-preserved human serum (Bioreclamation, Westbury, NY) and incubated in presence of AP/CP pathway-specific buffer diluent at 37 °C. To assess the contribution of the binding of CR2 to C3 fragments to the activity of TT32, the molecule was incubated with excess inhibitory (clones 171, 1048) or non-inhibitory (clone 629) anti-human CR2 mAbs. Aliquots of complement-preserved normal serum from each species (representing 100% activity) and heat-inactivated complement-depleted pooled normal serum (representing 0% activity), were used as positive and negative controls for the assay, respectively. The amount of complement activation is proportional to absorbance at 405 nm as determined with an ELISA Plate Reader (BioTech® Instruments). GraphPad Prism 5 software was used for curve fitting and the estimation of the IC_{50} values.

2.4. Rabbit red blood cell (RBC) AP hemolysis assay

This assay measures the release of hemoglobin from rabbit RBCs lysed upon exposure to AP-activated serum. Double distilled water lyses RBCs and was used as the positive control for lysis. EDTA (42 mM) chelates Mg^{2+} ions required for AP convertase activity and was used as a negative control for lysis. This assay was used for assessing TT32 activity, i.e., the extent to which TT32 inhibits AP-mediated hemolysis of rabbit RBCs. As serum C3 is activated, C3 convertases, C3 activation fragments and C5 convertases are deposited on rabbit RBCs. Serum AP activity in the presence of TT32 was evaluated in a concentration-dependent manner. Rabbit RBC (Bioreclamation, Westbury, NY) were washed, adjusted to 2.9×10^9 erythrocytes/mL and incubated with complement-preserved human or mouse (strain C57Bl/6 J) serum (50–80%) containing serial dilutions of TT32, TT30, CR1 1–10, inhibitory (clone 171) or non-inhibitory (clone 629) anti-mouse CR2 under experimental conditions promoting AP activity (Mg-EGTA) and subsequent hemolysis. After 30 min at 37 °C, 25 mM EDTA was added to stop the reaction, followed by centrifugation and removal of the supernatant to a new plate that was read at 415 nm to quantitate the released hemoglobin. Percent lysis was calculated as $(A415_{sample}\text{-}A415_{negcontrol})/ (A415_{H20})*100$.

2.5. Sheep red blood cell (SRBC) CP hemolysis assay

This assay measures the release of hemoglobin from sensitized sheep RBCs lysed upon exposure to CP-activated serum. Double distilled water and assay buffer were used as positive and negative controls, respectively. TT32 activity was accessed as the extent to which TT32 inhibits CP-mediated hemolysis of sheep RBCs (SRBCs). Sensitization of sheep RBC (Bioreclamation, Westbury, NY) was achieved by incubation with rabbit anti-sheep hemolysin (Cedarlane Labs) in the presence of gelatin-veronal buffer (Boston Bioproducts) for 30 min at 37 °C. Next, the optimal serum concentration to lyse erythrocytes via CP activation was determined by incubating sensitized SRBCs with serial dilutions of complement-preserved human serum and measuring hemoglobin release at 541 nm. Lastly, TT32 and other inhibitors were spiked into serum at the dilution determined as described above, and incubated with sensitized SRBC, followed by washing step and reading the absorbance at 541 nm. Percent lysis was calculated as $(A541_{sample}\text{-}A541_{negcontrol})/(A541_{H20}\text{-}A541_{negcontrol})*100$. All data calculations and analyses were performed using Microsoft Office Excel 2007 (Microsoft Corp.) and GraphPad Prism version 5. Serum AP and CP activities were reported as % activity (Sample – Neg. Control/(Pos. Control – Neg. Control) x 100 and normalized to the activity in serum.

2.6. Mice

Eight to 10-week-old Mice male DBA/1LacJ mice were used for pharmacokinetic and collagen-induced arthritis (CIA) studies. Eight to 10-week-old male C57BL/6mice were used for collagen antibody-induced arthritis (CAIA) studies. All CIA and CAIA studies used age-matched and sex-matched mice. All mice were obtained from the Jackson Laboratories. All animals were kept in a barrier animal facility with a climate-controlled environment having 12-h light/dark cycles. Filter top cages were used with 3 mice in each cage. During the course of this study, all experimental mice were fed breeder's chow provided by the Center for Laboratory Animal Care, University of Colorado School of Medicine.

2.7. Pharmacokinetics of TT32 in mice

DBA/1LacJ mice (n = 44, male, 8–10 weeks old) were dosed with 0.5 mg human TT32 per mouse either as single i.v., i.p. or s.c. injection. Intravenous injection was administered via a tail vein (i.v.), intraperitoneal injection (i.p.) was administered into the peritoneum, and subcutaneous injection (s.c.) was administered into loose skin over the shoulders. Serum was harvested at various time points (0.25, 0.5, 1, 2, 4, 8, 24, 48, 72 and 96 h) and stored at -80 °C until assayed for human TT32 concentrations. Serum concentrations of human TT32 were assessed by MSD immunoassay using anti-human CR1 Ab as capture and

anti-human CR2 as detection Ab.

2.8. Induction of arthritis in mice

2.8.1. Collagen antibody-induced arthritis

CAIA was induced in C57BL/6 mice by using a cocktail of 4 mAbs to bovine CII (Arthrogen-CIA, Chondrex) suspended in sterile Dulbecco's PBS. All 4 mAbs (3 IgG2a and 1 IgG2b) in this cocktail recognize conserved epitopes within the CB11 fragment, a recognition sequence that is shared by CII in many species. All mice received i.p. injections of 8 mg/mouse of Arthrogen on day 0 and 50 μg/mouse of LPS from *E. coli* strain 0111B4 on day 3 to synchronize the development of arthritis according to the methods described earlier (Banda et al., 2006). Three age-matched mice without inducing CIA were used as controls. All mice started to develop arthritis at day 4 and were sacrificed at day 10. The total number of mice used for CAIA studies was 24.

2.8.2. Collagen-induced arthritis

Bovine collagen type II (CII) (Elastin Products, Owensville, MO) was diluted in 0.01 M acetic acid to a final concentration of 4 mg/ml stored at -80 °C. An equal volume of CII was emulsified with Freund's incomplete adjuvant (IFA) (Difco, Detroit, MI) containing 4 mg/ml of inactivated *Mycobacterium tuberculosis* (H37Ra) (Difco, Detroit, MI). The emulsion was kept on ice during both preparations and use. Sixty DBA/1LacJ mice were immunized by intradermal injection (ID) at the base of the tail with 100 μl of the emulsion containing 200 μg of CII and 200 μg of *M. tuberculosis* in IFA according to our previously described methods (Banda et al., 2002). A booster injection was administered ID on day 21. Three age-matched, non-immunized DBA lac1/j mice were used as controls. Sixteen mice were sacrificed at day 27, and forty-two mice were sacrificed at day 35. The total number of mice used for CIA studies was 98.

2.9. Examination for clinical disease activity in mice with CAIA and CIA

The prevalence of disease and severity of clinical disease activity (CDA) in mice with CAIA was determined every day from day 4 to day 10 by a trained individual blinded to the experimental treatment group. All mice with CAIA were sacrificed at day 10. The prevalence of disease in mice with CIA was examined every other day from day 23 to day 35. The CDA score is based on a 3 point scale per paw: 0 = normal joint; 1 = slight inflammation and redness; 2 = severe erythema and swelling effecting the entire paw with inhibition of use; and 3 = deformed paw or joint with ankylosis, joint rigidity and loss of function (Banda et al., 2006). The total CDA score is based on all 4 paws with a maximum score of 12 for each mouse both in CAIA and CIA studies.

2.10. Treatments in CAIA studies

This experiment was done using C57 BL/6 WT mice (n = 24) to examine the effects of TT32 and anti-mouse C5 clone BB5.1 (Wang et al., 1995) on CAIA. In this experiment, three groups of 7 mice each were used in addition to three untreated control mice. Mice in each treatment group were injected i.p. three times on predetermined days with either Dulbecco's phosphate buffered saline (PBS), TT32 or anti-C5 mAb. The i.p. injection volume was kept constant at 400 μl/mouse. The first group was injected with PBS 15 min after the injection of 8 mg of Arthrogen at day 0, and again 15 min after the injection of LPS at day 3 followed by another injection at day 6. The second and third groups were injected on days 0, 3 and 6 with 2 mg of TT32/mouse and 0.750 mg/mouse of anti-C5 antibody respectively. All mice treated with sterile PBS, TT32 and anti-C5 mAb started to develop disease at day 4 and were sacrificed at day 10. Blood was drawn retro-orbitally from all mice at day 0 before injection of mAb to CII, at day 3 before LPS injection and at day 10 before sacrifice. All procedures were approved by the Institutional Animal Care and Use Committee of the IACUC of the University of Colorado.

2.11. Treatments in CIA studies

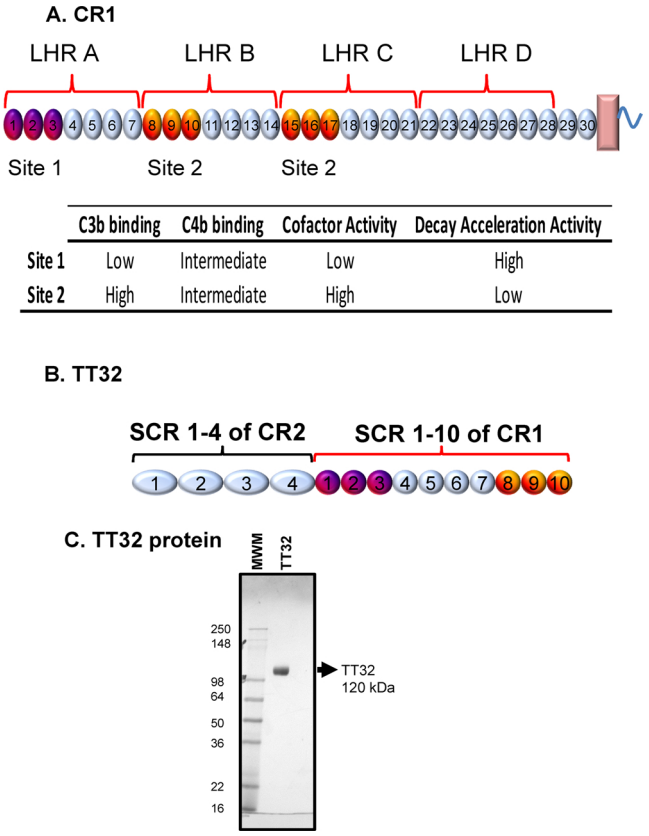
There were two parts to this study. The first part of the study was done with DBA/1LacJ mice comparing the frequency of TT32 injections; keeping the dose constant to 2 mg/mouse during the course of experiment. After the second injection of CII on day 21, mice were randomly divided into four treatment groups of 7 mice each. Thirty-three mice in total were used for this study, including five control mice (i.e. without the induction of disease or treatment). The first group was injected i.p with 200 μl/mouse of PBS (3x per week). The second group was injected i.p with 200 μl/mouse of 2 mg TT32 (1x per week i.e. 1 IN/WK). The third group was injected i.p. with 200 μl/mouse of 2 mg TT32 (2x per week i.e. 2 IN/WK). The fourth group (n = 7) was injected i.p. with 200 μl/mouse of 2 mg of TT32 (3x per week i.e. 3 IN/WK).

The second part of the study was done to compare the effects of the equivalent molar (nmol) amounts of TT32 and sCR1–10 on CIA. Three different amounts of TT32 were used: 17.46 nmol (2 mg/mouse), 6.55 nmol (750 μg/mouse) and 2.18 nmol (250 μg/mouse). The same molar concentrations of sCR1–10 were also used (i.e. 17.46 nmol (1.58 mg/mouse), 6.55 nmol (594 μg/mouse) and 2.18 nmol (198 μg/mouse)). For the anti-C5 mAb, based on our previous experience, an intermediate 6.55 nmol (1.24 mg/mouse) concentration was used. Anti-C5 treatment was used as a positive control in this study. After the second injection of CII on day 21, mice were randomly divided into eight treatment groups (n = 7 each). In total, 61 mice were used for this study, including 5 controls (without the induction of disease and without any treatment). All mice were only treated at day 21 (i.e. one hour after booster injection with 200 μl/IP of TT32, sCR1–10 or anti-C5 ab).

To examine the effect of TT32 on C3 deposition and levels after treatment in the knee joints, and in the circulation respectively, two mice from each group were sacrificed at day 27. Both forepaws and the right hind limb (including the paw, ankle and knee) were surgically removed from 16 mice at day 27 and joints were fixed in 10% neutral buffered formalin (NBF) (Biochemical Sciences, Swedesboro, NJ). Serum from each mouse was obtained by retro-orbitally aspiration of blood at day 0 before CII injection, at day 21 before booster injection and at again day 35 before sacrificing according to the methods described (Banda et al., 2012; Lachmann, 2010). Each sample was analyzed in triplicate by enzyme-linked immunosorbent assay (ELISA) specific for IgG1 and IgG2a antibodies to CII (Caltag, Burlingame, CA) using our published methods (Banda et al., 2002; Willis et al., 2011). To examine the direct effect of TT32, sCR1–10 and anti-C5 on weight loss or gain, during the course of treatments, all mice were weighed prior to CII injection, prior to booster injection and again, before sacrificing at day 35.

2.12. Histopathology and C3 deposition

Both forelimbs and the right hind limb, with knee joint, ankle and paw, from C57 BL/6 mice with CAIA and DBA/1LacJ with CIA at day 10 and at day 35, respectively, were fixed in 10% NBF to examine for histopathological changes and C3 deposition. Histopathology with scores for inflammation, pannus, cartilage and bone damage was assessed by using Toluidine-blue (T-blue) according to our published criteria (Banda et al., 2006). C3 deposition was assessed by using a primary polyclonal goat anti-mouse C3 Ab (dilution 1:10,000) (ICN Pharmaceuticals, Costa Mesa, CA) and detected by goat anti-HRP polymer kit as per manufacturer's instructions (Biocare Medical, Concord, CA). Visualization of reactivity against C3 protein was carried out using 3′, 3′ diaminobenzidine solution substrate (DakoCytomation, Carpinteria, CA) which reacts with HRP and produces a brown color stain.



	C3b binding	C4b binding	Cofactor Activity	Decay Acceleration Activity
Site 1	Low	Intermediate	Low	High
Site 2	High	Intermediate	High	Low

Fig. 1. A. Schematic domain structure of CR1. CR1 (CD35) consists of 30 short consensus repeats (SCRs, ovals), transmembrane domain (rectangle) and a short cytoplasmic region (blue tail). Each of four long homologous repeats (LHRs A–D) consists of seven SCRs and the complement regulation activity is localized in first three SCRs of each LHR, called sites. There are two distinct functional sites in CR1 – site 1 (LHR A) possessing intermediate affinity for C4b and displaying mostly decay acceleration activity while site 2 (LHR B and C) exhibits high affinity for C3b and functions as a cofactor for factor I. B. Composition of TT32. First four SCRs of CR2 are joined to first ten SCRs of CR1. The CR2 region is responsible for binding to C3dg/C3d and iC3b, while first ten SCRs of CR1 inhibit both AP and CP via decay-acceleration and cofactor activity. C. TT32 was purified using a three step process, a mixed mode capture resin, followed by butyl sepharose, and lastly polished using cation exchange (Resource S). One μg of purified TT32 was loaded onto a 4–20% Tris-glycine gel and stained with Comassie blue to visualize a single band at 120 kDa.

2.13. Statistics

To find out the normality of the data, w-statistics were used. Where data were not normally distributed, the Mann-Whitney test was used. Student's *t*-test was used to calculate *p*-values, with the GraphPad Prism® 4 statistical program. The data in graphs, histograms and tables are shown as the mean + SEM, with $p < 0.05$ considered significant. One way analysis of variance (ANOVA) using Tukey's multiple comparison test was also performed to further confirm the significant differences regarding clinical disease activity (CDA) scores among various treatment groups of CAIA and CIA mice, including PBS, TT32, SCR1–10 and anti-C5 ab.

3. Results

3.1. Design and expression of TT32

TT32 is a 901-amino acid, 120 kDa recombinant human fusion protein comprising SCRs 1–4 of CR2 and SCRs 1–10 of CR1 (Fig. 1A, B & C). We directly joined the sequences of CR2 and CR1 without introducing any linker sequences. However, the SCR domains 3 and 4 of CR2 serve a spacer function, as they are not required for the binding of CR2 to iC3b, C3dg or C3d. TT32 was expressed in human embryonic kidney 293 F cells and purified to homogeneity via a series of standard chromatography separations.

3.2. Binding affinity of TT32 to C3d and C3b by Biacore

The Biacore binding sensorgrams and steady state equilibrium analysis for the binding of TT32 to C3b and C3d are provided in Fig. 2. TT32 was shown to bind to C3b with an equilibrium dissociation constant (KD) of 4.94 μM (Fig.2A-B). The K_D for TT32 binding to C3d was similar and determined to be 3.7 μM (Fig. 2C-D).

3.3. Activity, selectivity and potency of TT32

We assessed the AP and CP inhibitory activity of TT32 using the Wieslab Complement System Alternative and Classical Pathway ELISA kits (Fig. 3). AP and CP inhibition was measured after addition of TT32 to pooled human serum (Fig. 3A, 3B). TT32 is a potent inhibitor of both AP (IC_{50} = 11 nM) and CP (IC50 = 46 nM), and its inhibitory activity for both pathways is more potent compared to the soluble CR1 composed of CR1 SCR domains 1–30 (sCR1; AP IC_{50} = 20 nM, CP IC_{50} = 250 nM). It is notable that the AP inhibitory activity of TT32 is greater as compared to the previously created CR2-targeted AP inhibitor TT30 (AP IC_{50} = 130 nM). Noting that sCR1 contains an additional site 2 in the LHR C, we constructed a truncated version of sCR1 containing only SCRs 1–10 (CR1 1–10) and compared this untargeted minimal construct with TT32 (Fig. 3C, D). The untargeted CR1 1–10 is an approximately twenty-fold weaker inhibitor of the AP (IC_{50} = 210 nM) and an approximately ten times weaker inhibitor of CP (IC_{50} = 450 nM) when compared to TT32.

To determine the inhibitory activity of TT32 in the *ex-vivo* system, we employed the erythrocyte hemolysis assay in which the rabbit erythrocytes are exposed to AP-activated human serum, leading to C3 activation and ultimately C3 fragment deposition on the erythrocytes (Fig. 4). The following steps of the complement cascade result in C5 activation and ultimately MAC insertion into the cell membrane, resulting in cell lysis. TT32 was more potent inhibitor of erythrocyte lysis compared to TT30 in both human and mouse sera (TT32: IC_{50} = 240 nM in human, 75 nM in mouse sera; TT30: IC_{50} = 870 nM in human, IC_{50} = 540 nM in mouse sera (Fig. 4A) and the targeting efficacy of the CR2 module was shown when compared to CR1 1–10 (IC_{50} = 2100 nM (Fig. 4B).

In order to assess the CP inhibitory potential of TT32, the sheep erythrocyte CP lysis assay was performed as well (Fig. 4C). The results from the rabbit and sheep RBC lysis assays therefore confirm the results obtained from the Wieslab Complement System Alternative and Classical Pathway ELISAs.

The role of the targeted AP inhibition was assessed by spiking the TT32 into human serum containing the inhibitory anti-CR2 monoclonal antibodies (clone 171) or non-inhibitory anti-CR2 monoclonal antibody (clone 629) in the rabbit RBC lysis assay (Fig. 5A) and a similar result was obtained compared to the Wieslab AP ELISA (Fig. 5B).

3.4. Bioavailability and pharmacokinetics of TT32 in mice

The pharmacokinetics of TT32 was assessed in DBA/1Lacj mice dosed with 0.5 mg TT32 per mouse as a single i.v., i.p., or s.c. injection. Serum samples were harvested at various time points and assayed for TT32 concentrations by MSD immunoassay (Fig. 6). Intravenous administration of TT32 resulted in peak serum concentration of 230 μg/ml while i.p. and s.c. injections produced lower maximal serum concentrations (21 and 7 μg/mL). The terminal half-life of TT32 was estimated to be 2.6 h (i.v.), 2.5 h (i.p.) and 7.5 h (s.c.). The bioavailability of TT32 in mice was calculated as a ratio of areas under curve (AUC) as

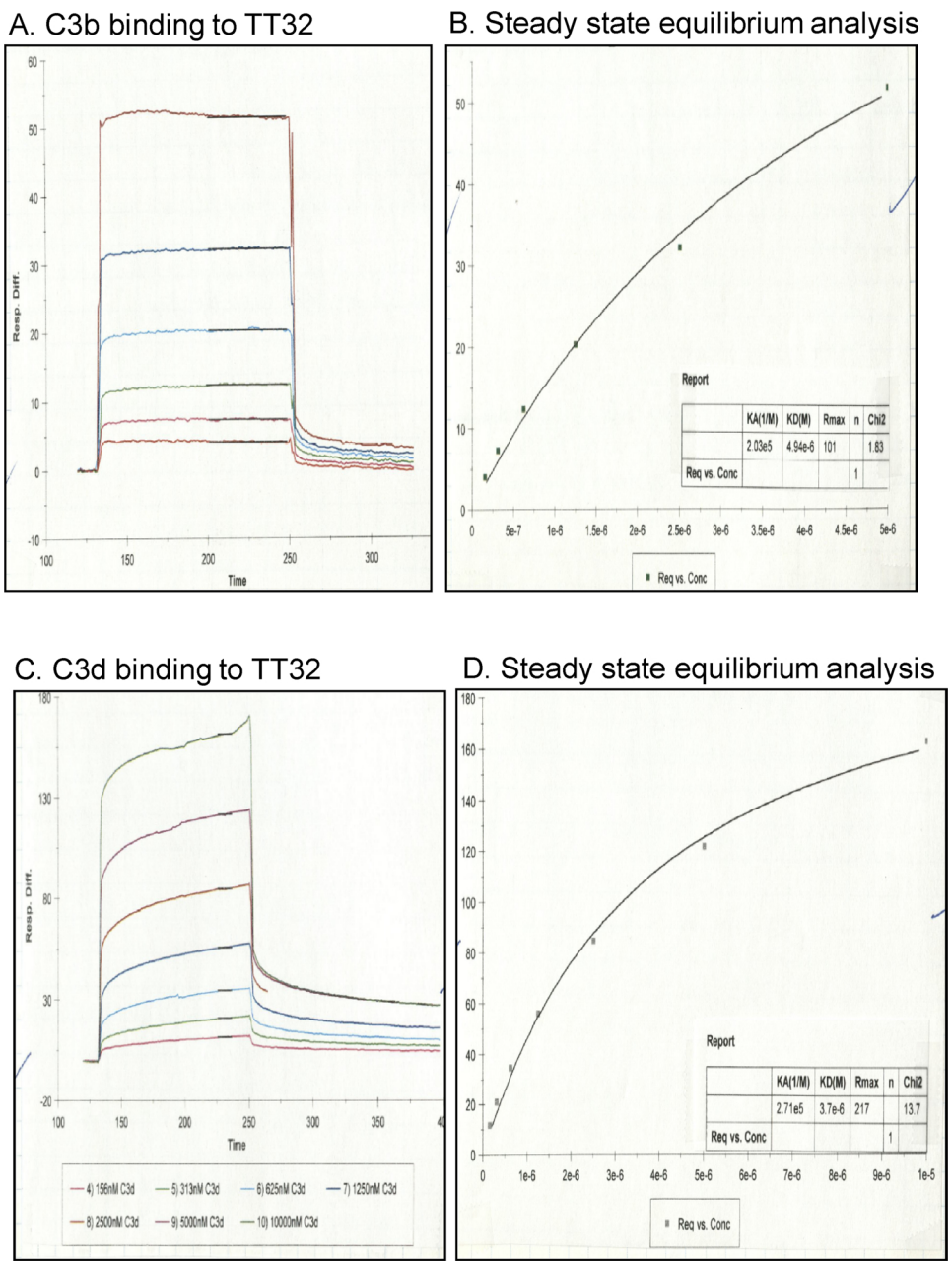


Fig. 2. Surface plasmon resonance sensorgrams demonstrating the association and dissociation of TT32 with ligands C3b and C3d. **A.** TT32 was immobilized on the CM5 chip using amine coupling. Different concentrations of C3b were injected as a solution phase analyte. The data was analyzed using steady state equilibrium analysis and K_D calculated (B). **C.** TT32 was immobilized on the CM5 chip using amine coupling. Different concentrations of C3d wereinjected as a solution phase analyte. The data was analyzed using steady state equilibrium analysis and K_D calculated (D). TT32 bound either C3b or C3d with a K_D of approximately 4 μM.

determined by non-compartmental analysis; the i.p. bioavailability of TT32 was 21% and the s.c. bioavailability was 29%. Interestingly, the half-life of s.c. injected TT32 was three times more than i.v. or i.p. injections.

3.5. Clinical disease activity in CAIA mice treated with TT32

To determine the effect of TT32 on CAIA, mice were treated with PBS (n = 7) TT32 (2 mg)(n = 7) and anti-C5 antibody (0.750 mg) (n = 7) for three days i.e. at day 0, day 3 and at day 6 as mentioned in Methods (Fig. 7). Anti-C5 mAb treatment of mice with CAIA was used as a positive control in this study based on previously published studies (Wang et al., 1995). CDA was examined every day from day 4 to day 10 by two observers who were blinded to the treatment. There was a significant ($p < 0.001$) decrease in the CDA, from day 4 through day 10, in mice treated with TT32 and anti-C5 mAb compared with mice treated with PBS (Fig. 7A).The average CDA at day 10 was 8.42 ± 0.812, 4.42 ± 0.369 and 4.0 ± 0.534 in mice treated with PBS, TT32 and anti-C5 mAb respectively (Fig. 7A). Thus, treatment with TT32 resulted in a 47.4% decrease in the CDA. No significant differences ($p = 0.53$) were seen at any day in the CDA between mice treated with TT32 and anti-C5 mAb (Fig. 7A).The prevalence of disease was 100% in all treatment groups at day 10 (Fig. 7B). However the prevalence at day 6 was ~70% in mice treated with TT32 and anti-C5 mAb and was 100% in mice treated with PBS alone (Fig. 7B).This study shows that treatment of mice with TT32 or anti-C5 significantly reduced the severity and delayed the onset of CAIA.

All mice were weighed (grams) at day 0 and at day 10. At day 0, the mean ± SEM weight was 24.3 ± 0.495, 23.5 ± 0.522 and 24.3 ± 0.864 in mice treated with PBS, TT32 and anti-C5 mAb

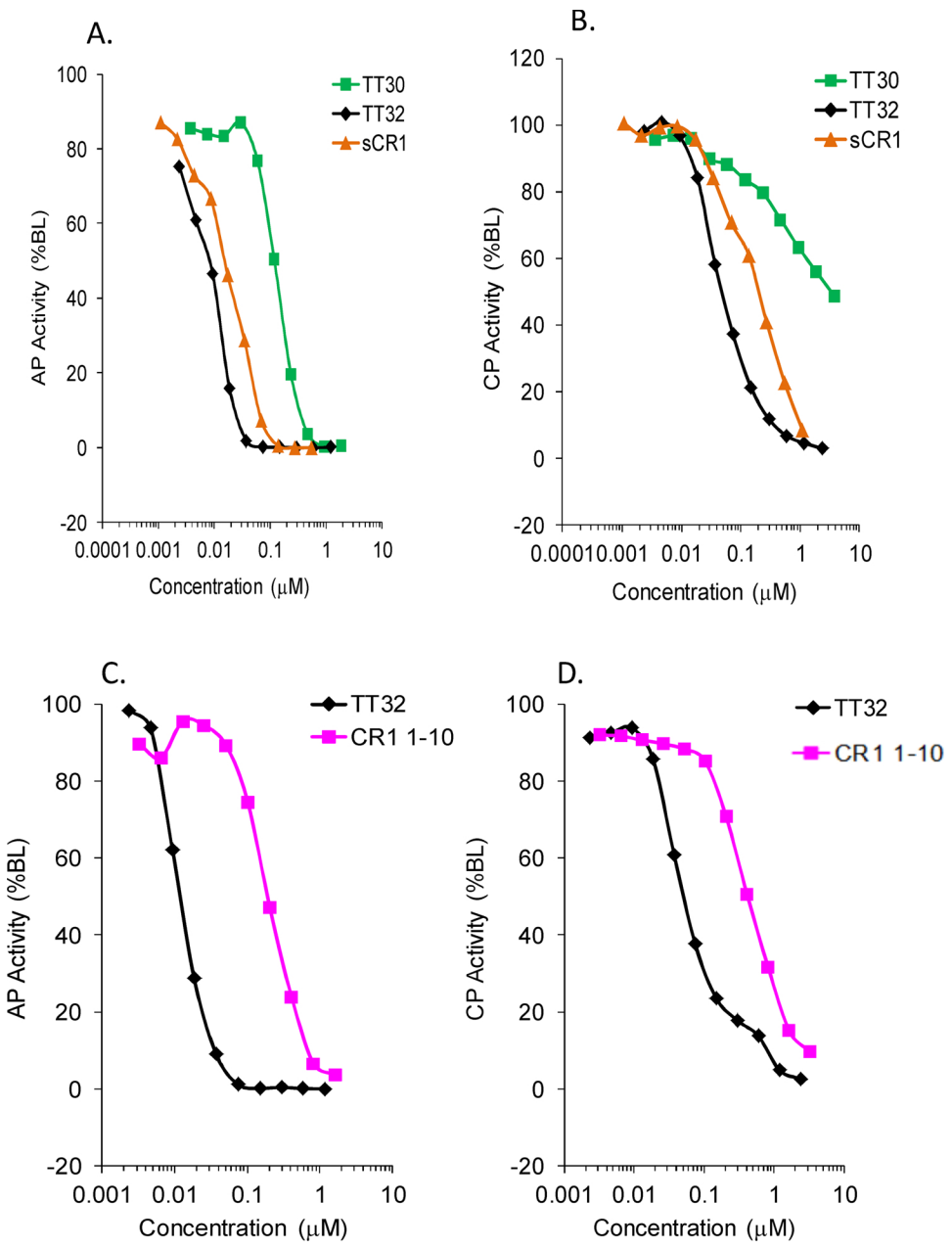


Fig. 3. Using Wieslab ELISA-based methods, CR2-CR1 (TT32) is a more potent alternative (A, C) and classical (B, D) pathway inhibitor than TT30, sCR1 or CR1–10 alone.
(A, B) The activity of TT30, TT32 and soluble CR1 (sCR1, SCR domains 1–30) was compared in both AP and CP Wieslab ELISA assays. TT32 is the most potent molecule in both assays (AP IC50 11 nM, CP IC50 46 nM) followed by sCR1 (AP IC50 20 nM, CP IC50 250 nM). TT30 is an AP-selective inhibitor (IC50 130 nM), inhibition of CP was only partial. (C, D) The role of the CR2 targeting moiety was analyzed next - since TT32 contains only first ten SCRs of CR1, Wieslab AP and CP inhibition by TT32 was compared to the inhibition by CR1 SCR 1–10 (CR1 1–10). TT32 is 20 times more potent than CR1 1–10 in the AP assay (TT32 IC50 11 nM; CR1 1–10 IC50 210 nM) and 10 times more potent in the CP assay (TT32 IC50 53 nM; CR1 1–10 IC50 450 nM).

respectively. At day 10, the mean weight was 22.9 ± 0.61, 23.3 ± 0.43 and 24.7 ± 0.61 in mice treated with PBS, TT32 and anti-C5 mAb respectively. There was no significant ($p = 0.73$) weight loss in mice before or after the treatment with TT32 or anti-C5 mAb. In contrast, a minor significant ($p < 0.046$) decrease in weight as expected was noticed in mice treated with PBS at day 10 vs. at day 0.

3.6. Clinical disease activity in CIA mice treated with TT32

To determine the effect of TT32 on CIA two different experiments were done. First, a CIA experiment was done to determine the overall efficacy of TT32 using a single dose of 2 mg/mouse but with varying injection schedule per week (i.e. from one i.p. injection to three i.p. injections/mouse) (Fig. 8).Fifteen minutes after the booster injection of CII, at day 21, mice were injected with TT32 (2 mg/mouse) once a week ($n = 7$), twice a week ($n = 7$) and three times a week ($n = 7$) (Fig. 8A). All groups of mice were randomized before treatment. The control group of mice was injected with PBS three times a week. CDA was examined three times a week from day 23 to day 35 by two observers who were blinded to the treatment. The CDA in mice, at day 35, injected with TT32 one, two and three times per week was 4.85 ± 1.05, 7.28 ± 1.22 and 4.28 ± 1.128 respectively (Fig. 8A).The CDA in mice injected with PBS was 10.0 ± 0.787. CIA mice injected with TT32 only once a week, from Day 23 to Day 35, showed a significant ($p < 0.05$) decrease in CDA compared with the CDA in PBS treated mice. CIA mice injected with TT32 three times a week were also protected from arthritis significantly ($p < 0.05$). No significant decrease in CDA was noticed with the twice weekly injections of TT32 compared with PBS treated mice (Fig. 8A). The prevalence of disease, at day 35, was 100% in mice treated with PBS (once per week) and with TT32 (twice per week) (Fig. 8B). The prevalence of disease at day 35 was 85% in mice treated three times per week with TT32 (Fig. 8B). There was no significant change in body weight in all groups of mice at day 10 (Fig. 8C).This efficacy study showed that mice injected with TT32 once or three times per week resulted in a significant ≥51.5% decrease in the CDA at day 35. However, CDA in the group of mice that received TT32 twice a week was reduced by approximately 25% at day 35.

The second CIA experiment was done to determine the effect of

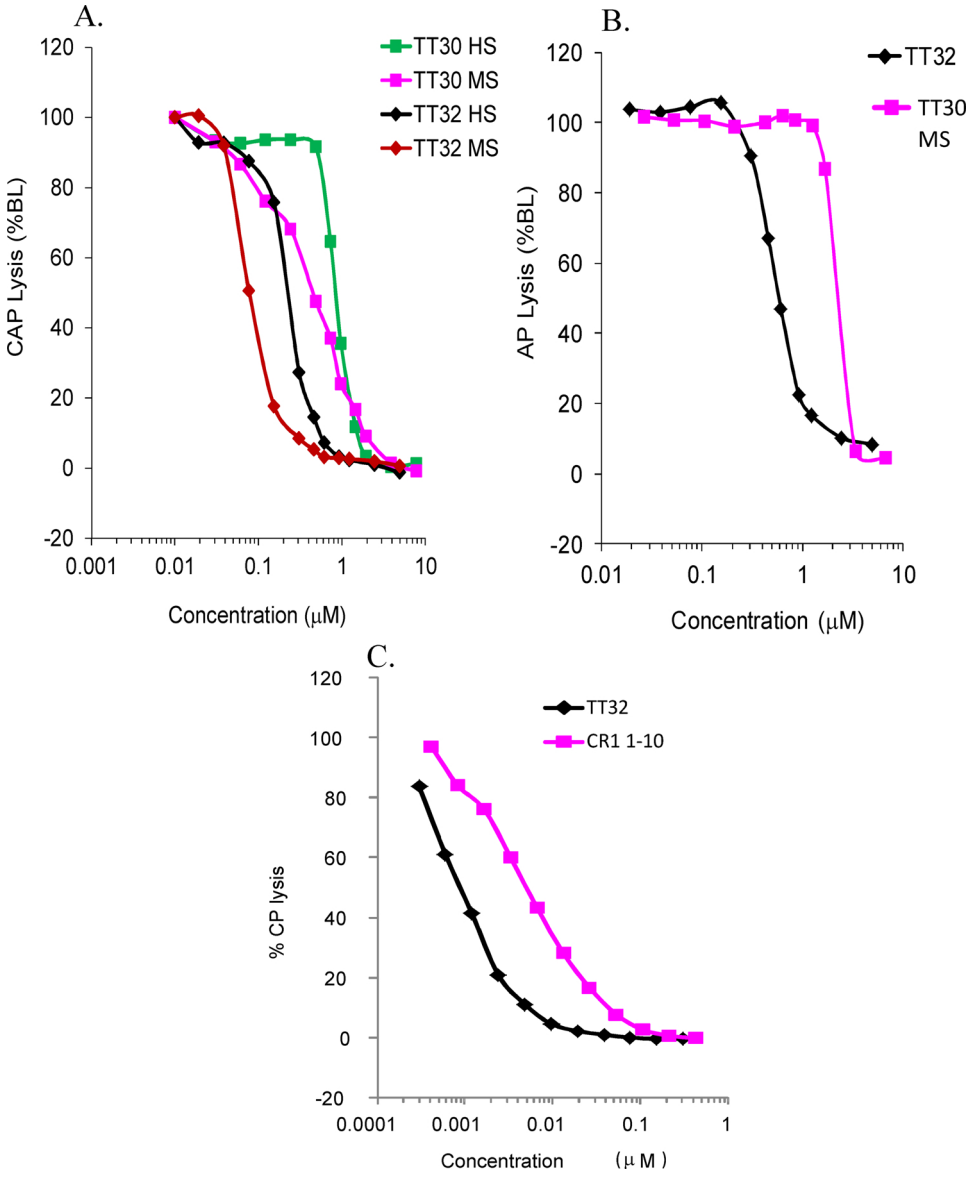


Fig. 4. Effect of TT32 on AP50 and CP50 activities. The AP50 and CP50 haemolytic complement activities were measured by using Rabbit blood cells (RBCs) and Sheep red blood cells lytic assays, respectively. The AH50 and CH50 were determined by analyzing the capacity of human and mouse serum incubated with various doses of TT32 or TT30 or SCR1–10 to lyse antibody coated RBCs or SBCs. The calculated percentage (%) of lysis of the control and serum samples from human and mouse were plotted against dilution factor. TT32 inhibits both human (A, B) and mouse (A) complement activation more efficiently than TT30 or CR1 1–10 using rabbit RBC alternative pathway (A, B) or sheep RBC classical pathway (C) lysis assay. HS, human serum; MS, mouse serum.

equivalent molar amounts of TT32 on CIA compared with the non-targeted inhibitor, sCR1–10 (Fig. 9). Fifteen minutes after the booster injection of CII, at day 21, mice were treated only one time with 400 μl/mouse i.p. of TT32 using 17.46 nmol (2 mg) (n = 7) or 6.55 nmol (0.750 mg) (n = 7) or 2.18 nmol (0.250 mg) (n = 7) and using sCR1–10 17.46 nmol (1.58 mg) (n = 7) or 6.55 nmol (0.594 mg) (n = 7) or 2.18 nmol (0.198 mg) (n = 7) (Fig. 9). At the same time, after a booster injection, two groups of mice were treated: one with PBS alone (n = 7) and a second with 6.55 nmol (1.24 mg) (n = 7) of anti-C5 mAb. The CDA was examined blindly three times a week as mentioned above. Importantly, the CIA mice treated once with 17.46 nmol of TT32 were protected by 60% and 52% from arthritis compared with mice treated with PBS and sCR1–10 (17.46 nmol), respectively (Fig. 9A). The CDA, at day 35, in mice treated with TT32 amounts of 17.46 nmol, 6.55 nmol and 2.18 nmol was 3.2 ± 1.24, 4.66 ± 0.95 and 5.8 ± 1.59 respectively (Fig. 9A, B & C). In contrast CDA, at day 35, in mice treated with sCR1–10 concentration of 17.46 nM, 6.55 nM and 2.18 nM was 6.6 ± 0.67, 7.4 ± 1.20 and 5.2 ± 0.37 respectively (Fig. 9A, B & C). The CDA, at day 35, in mice treated with PBS and anti-C5 mAb (6.55 nmol) was 8.0 ± 0.577 and 3.8 ± 1.39 respectively (Fig. 9A, B & C). No significant differences ($p = 0.14$) were seen in the CDA in mice treated with 17.46 nM and 6.55 nM of sCR1–10 vs. mice treated with PBS (Fig. 9A, B & C). Individual variations in the CDA of each mouse during the course of treatment have also been shown (Fig. 10A-E). As expected the CDA in mice treated with anti-C5 mAb was significantly decreased from day 23 ($p < 0.008$) through day 35 ($p < 0.015$) (Fig. 9). The prevalence of disease was 100% in all treatment groups at day 35 (data not shown). There was no significant change in weight in all treatment groups of mice from day 0 to day 35 (Fig. 11A, B & C).

3.7. Anti-collagen antibodies

The IgG1 and IgG2a antibody responses to CII in mice injected with TT32 one, two and three times a week were measured in sera obtained at day 0, day 21 and at day 35 (Fig.12). No anti-collagen antibodies were detected at day 0 (i.e. prior to the induction of disease) as expected. No significant differences were seen in the levels of IgG1 (Fig. 12A). The data were expressed as a change in OD values in each treatment group between day 21, prior to injections of TT32 and at day 35. A significant 74% decrease ($p < 0.05$) in the levels of IgG2a was seen in the CIA mice injected three times per week with TT32 compared with PBS treated mice (Fig. 12B).

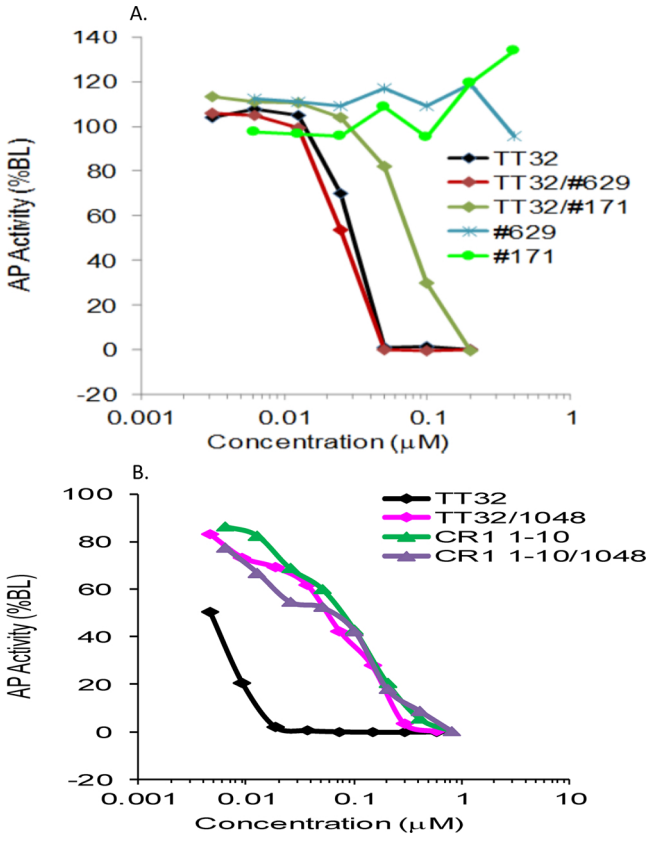


Fig. 5. CR2-mediated synergistic effect of TT32 on the AP and specific inhibition of synergistic effect by inhibitory mAbs. The AP50 haemolytic complement activity was measured by using RBCs lysis assay in Mg/EGTA buffer. The calculated percentage (%) of lysis of the control and serum samples from human were plotted against antibody concentration. A. CR2 targeting enhances TT32 potency against alternative pathway in rabbit RBC lysis B. Wieslab assay: comparison of effect of inhibitory (mAb 171, mAb 1048) versus non-inhibitory mAb (mAb 629) anti-CR2 antibodies.

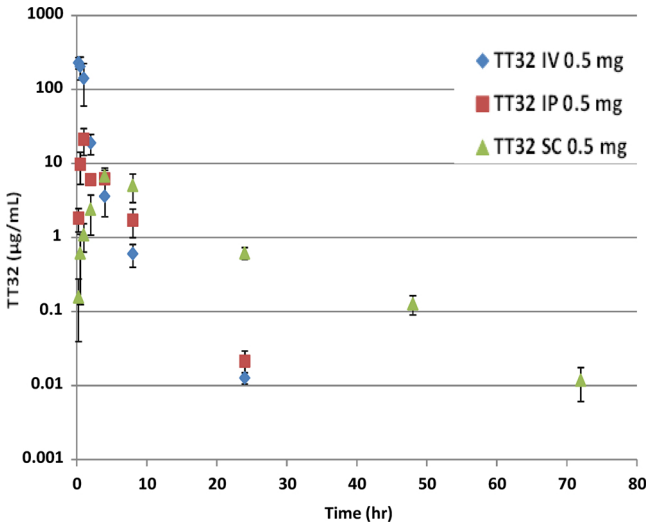


Fig. 6. Pharmacokinetics of TT32 in mice. DBA/1LacJ mice were injected (IV, IP & SC) with a single doses of 0.5 mg/mouse of TT32. Sera from these TT32 injected mice was examined at various time points (hr.) to confirm the bioavailability of TT32 in the circulation. TT32 is bioavailable in mice through IV, IP and SC routes of administration. Serum samples were examined at various time points shown in hours.

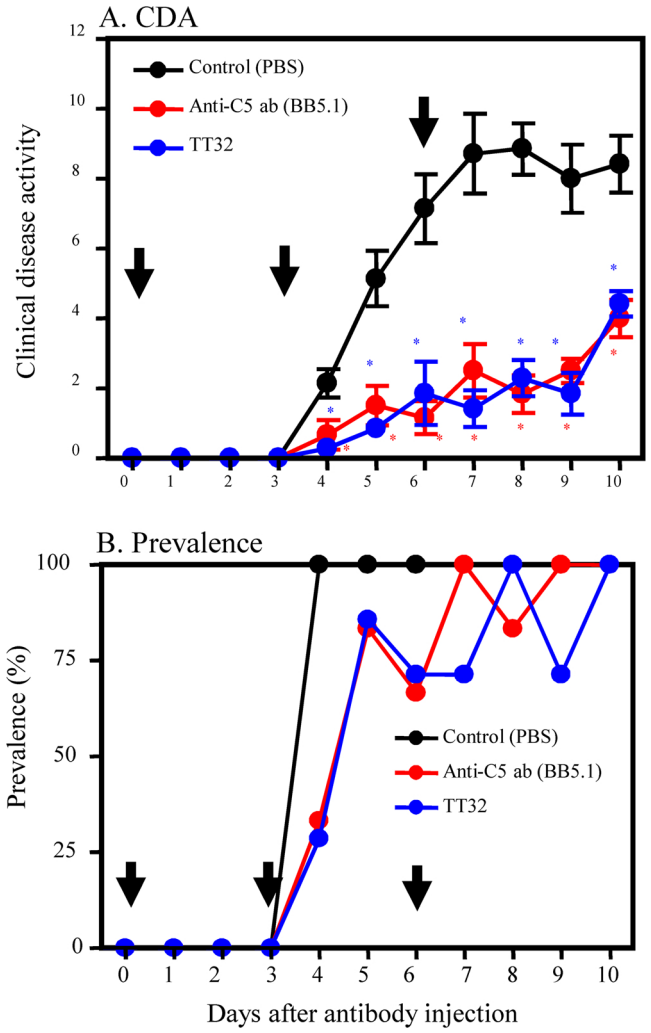


Fig. 7. Human TT32 attenuates CDA in mice with CAIA. CAIA in mice was induced by injecting (i.p.) with a mixture of anti-collagen antibodies (Arthrogen) and LPS according to the methods described in Methods section. Mice were injected (i.p.) three times at day 0, day 3 and at day 6 either with PBS or anti-C5 mAb or TT32. All mice were sacrificed at day 10. **A.** CDA in all joints over the duration of the experiment. **B.** Prevalence (%) in all mice over the duration of the experiment. Anti-C5 mAb (BB5.1) was used as a positive control in this CAIA study. Black arrows show the specific day mice were injected. The data represent the mean ± SEM for each group (n = 8). *$p < 0.05$ for TT32 or anti-C5a mAb in comparison to treatment with PBS only.

3.8. C3 deposition in the joints from mice with CIA

At day 35, C3 deposition in the joints of was measured by IHC analysis as described in Materials and Methods. At day 35, consistent with CDA and histology measurements (data not shown) there was a significant decrease in the C3 deposition in the synovium (87%) ($p < 0.041$), on the surface of the cartilage (97%) ($p < 0.048$) and total all-joint sum animal score (91%) ($p < 0.038$) in mice treated with TT32 (2 mg) compared with the mice treated with PBS alone (Fig.13). The decrease in the C3 deposition in mice treated with TT32 was greater than mice treated with sCR1 (Fig.13). Interestingly although all joints sum animal score (synovium and cartilage surface) for C3 deposition was reduced by 56% in mice treated with sCR1 (1.58 mg), it was not significant (Fig.13). There was also an overall decrease in the C3 deposition in mice treated with anti-C5 mAb (1.28 mg) in the synovium (65% reduction compared to PBS control) as well as on the surface of the cartilage (100% reduction compared to PBS control) (Fig.13).

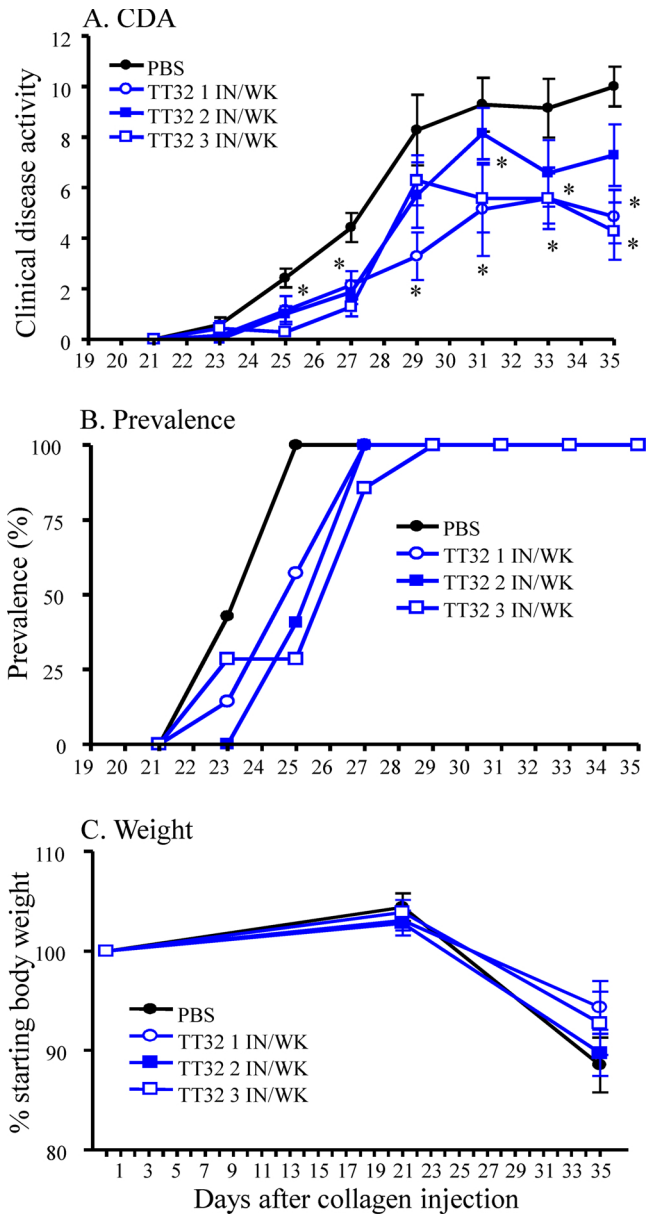


Fig. 8. A single dose of human TT32 per week protected mice from CIA with no observed toxicity. CIA was induced in mice by injecting intradermally (ID) on the tail using an emulsion of bovine type II collagen and *M. tuberculosis* at day 0 and at day 21 in IFA according to the procedures as described in detail in the Materials and Methods. A single dose of TT32 (2 mg/mouse) was injected in each mouse one hour after giving the second injection of bovine type II emulsion, starting at day 21. TT32 was injected in mice from day 21 through day 34 once, twice or 3 times per week. PBS treated mice were used as the control. All mice were sacrificed at day 35. **A.** CDA in all joints from day 21 through day 35. **B.** Prevalence (%) of disease. **C.** Percent change in weight during the experiment. The data represent the mean ± SEM for each group (n = 7). *$p < 0.05$ for TT32 in comparison to treatment with PBS using ANOVA as mentioned in the Methods section. IN/WK = number of injections per week.

4. Discussion

Dysfunctional complement regulators, persistent immune complexes and aberrantly glycosylated auto-reactive antibodies may lead to pathological complement activation through the AP, CP and LP. While complement activation associated with certain diseases such as PNH, aHUS, C3 glomerulonephritis and dense deposit disease is believed to be caused by dysregulation of the alternative pathway, complement activation associated with other diseases such as lupus nephritis and IgA nephropathy is believed to be initiated by excessive activation of the CP and/ or LP. Once complement activation has been initiated by the CP/LP, it may be further amplified by the AP. Therefore, it may be beneficial to treat certain complement-mediated diseases with inhibitors that target multiple activation pathways. However, complement activation may play both beneficial and harmful roles in certain pathological conditions. For example, in lupus nephritis it is believed that deposited immune complexes activate the CP and AP causing excessive C3 and C5 activation that may contribute to renal damage. However, efficient clearance of immune complexes requires the function of early components of the CP, and deficiencies in C1q and C4 predispose individuals to lupus (Botto et al., 2009). It is therefore important to understand where best to intervene with inhibitors of the complement system as it relates to individual diseases and pathological processes. To that end we sought to develop experimental systems in which inhibition of individual versus multiple complement pathways could be evaluated in models of complement-mediated disease. Building on the design of the targeted AP inhibitor TT30 (CR2-fH) as well as prior studies of the murine orthologues CR2-fH and CR2-Crry, we developed TT32 (CR2-CR1) which retains the targeting domains of CR2 and, in place of the AP inhibitory domains of factor H, contains the AP/CP/LP inhibitory domains of CR1. Thus the AP specific inhibitor TT30 and the AP/CP/LP inhibitor TT32, both of which function at the level of C3 and C5 convertase activation, can be evaluated in a system in which anti-C5 mAb and other complement inhibitors have previously demonstrated efficacy. This experimental system has the potential to further extend our understanding of how the individual complement pathways contribute to tissue damage and may help guide the design of human complement inhibitors that are tailored to specific diseases (Fig. 14).

TT32 was found to have greater *in vitro* potency than TT30 in Wieslab AP and CP ELISAs and rabbit (AP - specific) as well as sheep (CP - specific) erythrocyte lysis assays. It was clearly shown that the targeting CR2 moiety significantly contributes to the *in vitro* potency of TT32, since untargeted CR1 SCR 1–10 exhibited markedly lower potency in all assays tested. Additionally, the monoclonal antibodies neutralizing the CR2 functionality (mAb clones1048, 171) reduced the potency of TT32, while CR2 non-neutralizing monoclonal antibody 629 did not have any discernible effect. We used both the CIA model (active immunization) and also the CAIA (passive immunization) mouse models of inflammatory arthritis to determine the efficacy of TT32 *in vivo*. These data demonstrate that TT32 administration led to a marked reduction in the clinical disease activity in both CIA and CAIA models. Here mice were treated prophylactically with TT32 based on the findings that the deposition of C3 in the synovium and on the cartilage surface increases dramatically during the initiation of disease followed by stabilization (Banda et al., 2013). These *in vivo* results confirm the direct involvement of complement in these models, and demonstrate that TT32 is superior to non-targeted sCR1 (Fig. 9). We and others have shown that other fusion proteins such as CR2-fH and CR2-Crry ameliorated CAIA and CIA (Banda et al., 2009; Song et al., 2007). Although not a complement receptor, Crry is a functional analog of human CR1 with regard to its ability to regulate the mouse CP and AP (Bao et al., 2003), and like human CR1, can disrupt AP and CP C3/5 convertases The present work builds upon previous *in vivo* studies showing the efficacy of targeted delivery of CR2-Crry in models of complement-mediated diseases by demonstrating that similar efficacy is achieved with human CR1 fused to the targeting domains of CR2 (Holers et al., 2013). TT32 *in vitro* showed potent inhibition of the CP and AP. Previous studies have shown that both CP and AP are involved in CIA (Banda et al., 2006; Hietala et al., 2002). CAIA is specifically dependent on the AP and mice lacking fB and fD are resistant to the CAIA (Banda et al., 2010, 2006). Since TT32 administration affected a decrease in the clinical scores and histological in CAIA, it is likely that TT32 was able to inhibit the AP (i.e. C3 deposition) *in vivo* as it evident from the decrease in C3 deposition in the joints. Considering that properdin acts as a

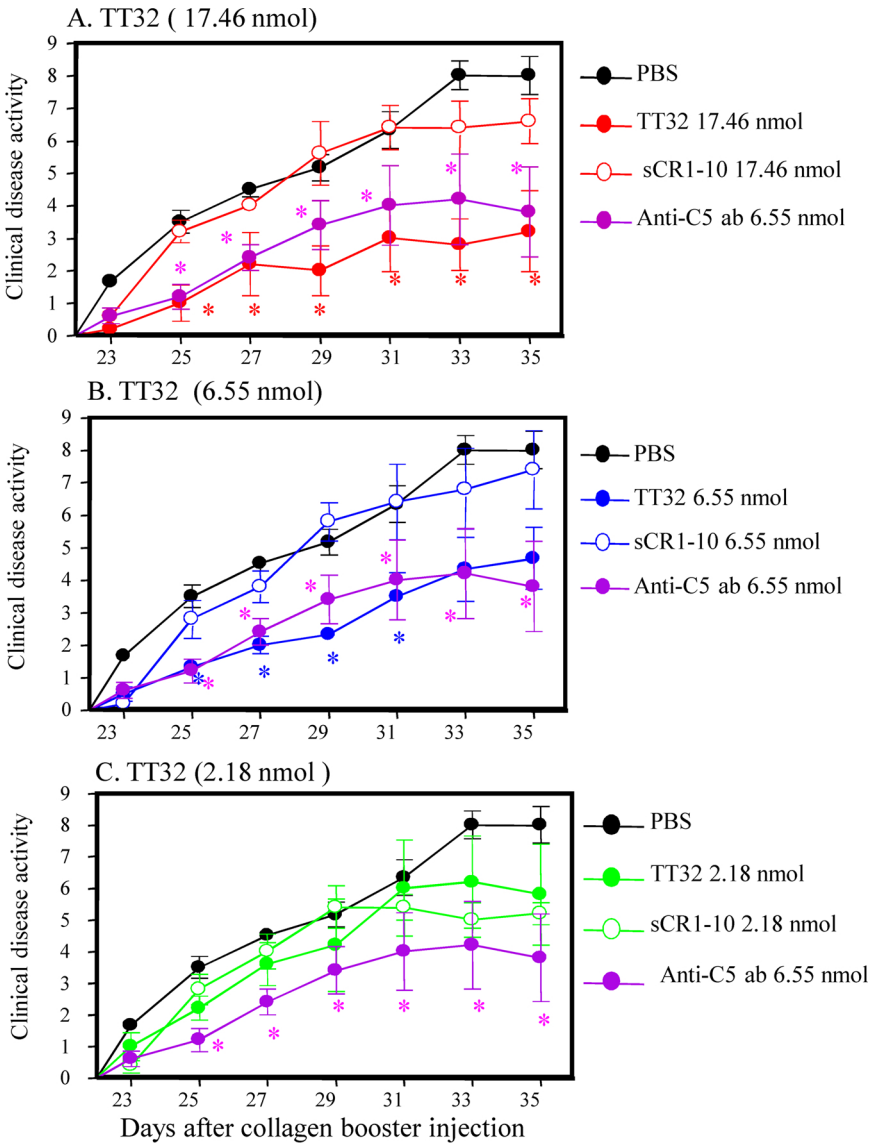


Fig. 9. Two different equivalent nanomolar (nmol) concentrations of human TT32 were superior compared to human sCR1–10 alone in protecting mice from CIA. CIA was induced in mice by injecting intradermally (ID) on tail using an emulsion of bovine type II collagen and *M. tuberculosis* at day 0 and at day 21 in IFA according to the procedures described in detail in the Materials and Methods. TT32 or sCR1–10 or PBS was injected (i.p.) only one time in each mouse, at day 21, one hour after giving a booster injection of the emulsion. Three different concentrations of TT32 in this CAIA were used and these were 17.46 nmol, 6.55 nmol and 2.18 nmol. Similarly, in parallel, mice were also injected (i.p.) with three different equivalent nano molar concentrations of the sCR1–10. Mice injected with PBS or anti-C5 mAb (6.55 nmol) served as positive and negative controls respectively. All mice were sacrificed at day 35. **A.** CDA in mice with CIA injected with 17.46 nmol of TT32 or sCR1-9. **B.** CDA in mice with CIA injected with 6.55 nmol of TT32 or sCR1-9. **C.** CDA in mice with CIA injected with 2.18 nmol of TT32 or sCR1-9. The data represent the mean ± SEM for each group (n = 5) except for TT32 6.55 nmol (n = 6). *$p < 0.05$ for TT32 in comparison to treatment with PBS only.

positive regulator of the alternative pathway by stabilizing the C3bBb convertase, it would be of value to determine if the activity of properdin was affected by TT32. For instance, properdin binding to complement activating surfaces was shown to be dependent on initial C3b deposition (Harboe et al., 2017) and properdin has been shown to bind directly to C3b (Kouser et al., 2016). It is conceivable that properdin and the CR2 domain of TT32 compete for binding to C3b. A component of the mechanism of action of TT32 may therefore be prevention of properdin binding to C3b, blocking its ability to stabilize the AP convertase.

TT32 was shown to bind to C3b and C3d in vitro. However, whether TT32 can target C3b or C3d *in vivo* under inflammatory conditions was not specifically determined in this study, and offers opportunity for further work. TT32 targeting *in vivo* may be addressed by administering labeled TT32 in CIA/CAIA and demonstrating the co-localization of TT32 with C3 fragments deposited within inflamed joints. Considering that TT32 was found to be more potent that the non-targeted sCR1 in vivo it is reasonable to interpret this increased potency to be due to the targeting activity of the CR2 domain of TT32. Thus TT32 has the potential to specifically localize to the inflammatory sites as it inhibited disease both in CIA and CAIA mouse models. Although not directly evaluated, the durable effect of a single dose of TT32 suggested that the molecule, like mouse CR2-Crry, was both targeted to the inflamed joint and retained at the site for a sufficient time to exert its protective effects.

We have not been able yet to perform studies wherein radiolabeled TT32 and sCR1 were administered in the CIA model to determine if the CR2 targeting domain of TT32 causes the compound to be localized to complement-opsonized inflamed joints. Such a study may provide insight to the mechanism by which TT32 had a clinical benefit while sCR1 did not. Studies have been completed with radiolabeled CR2-Crry, a murine orthologue of TT32, where localization of CR2-Crry within inflamed joints in CIA was correlated with clinical efficacy (Song et. al., 2007). Thus, our conclusions regarding the role of CR2 and the need for tissue targeting to see benefit are based on conclusions from the study with radiolabeled CR2-Crry referenced above as well as the study presented here where TT32 treatment significantly limited the clinical disease score in CIA while the non-targeted sCR1 did not.

It would have been of value to determine the effect of TT32 or sCR1 on the levels of C3 in the circulation of the disease induced mice. However, this is complicated by the use of LPS in the CAIA model, which was included to increase inflammation and disease severity. Given that LPS is both an activator of the AP, potentially leading to the consumption of C3, and an inducer of C3 expression (Cunningham et al., 2000) it would be difficult to ascribe changes in systemic C3 levels to TT32 or LPS in this model.

T lymphocytes have been implicated in sparking the inflammatory

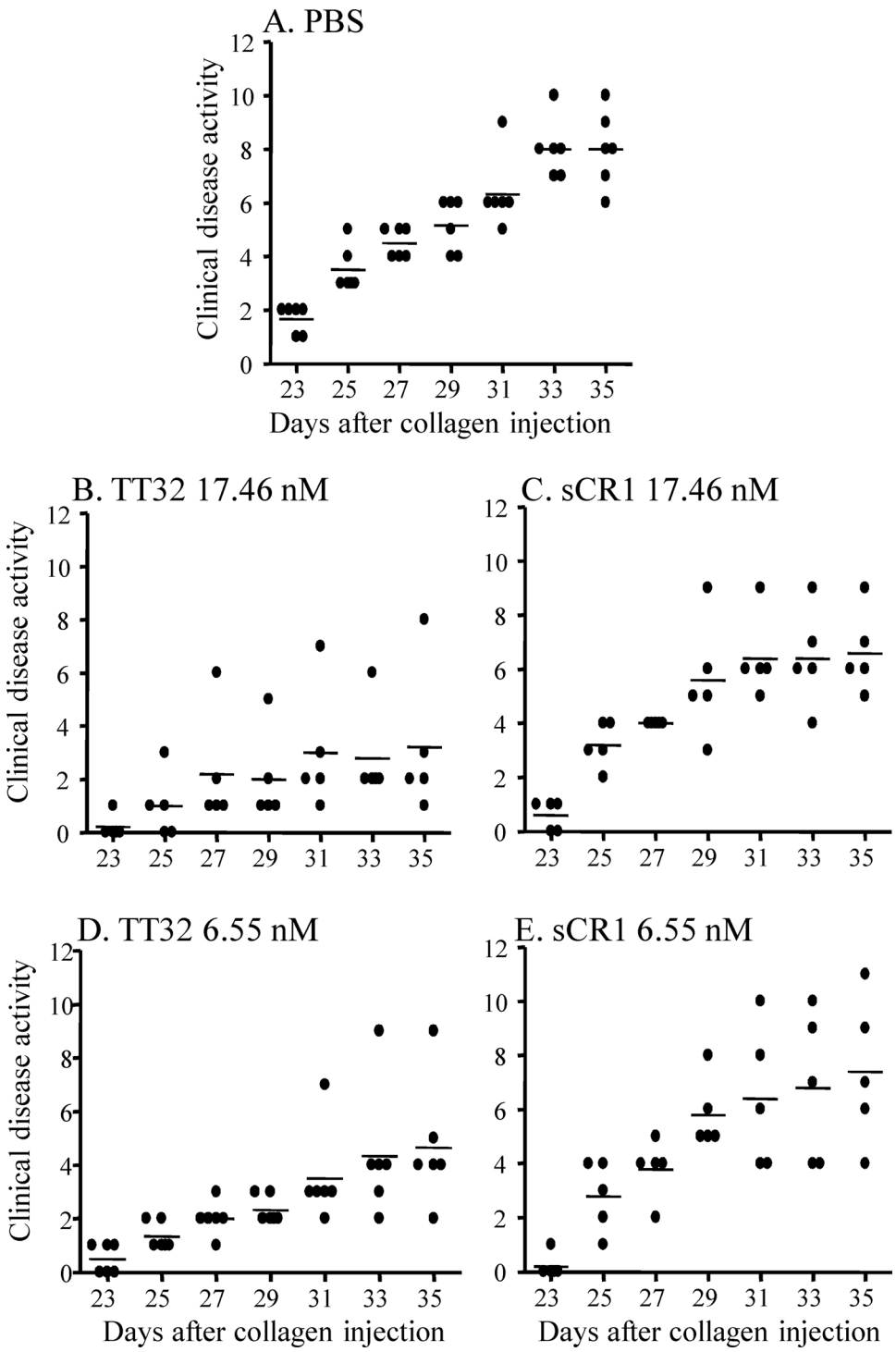


Fig. 10. Human TT32 at two different equivalent concentrations decreased the severity of disease in all mice in a cohort. A snapshot of the CDA in each mouse during the course of disease treated with two different equivalent molar concentrations of TT32 or sCR1–10. Disease was examined in each mouse every other day starting from day 21 through day 35. Each solid black dot represents an individual mouse. All mice were sacrificed at day 35. **A.** PBS treated mice. **B.** Mice treated with a dose of 17.46 nm of TT32. **C.** Mice treated with a dose of 17.46 nm of sCR1–10. **D.** Mice treated with a dose of 6.55 nm of TT32. **E.** Mice treated with a dose of 6.55 nm of sCR1–10. Maximum and minimum clinical disease activity score for each mouse from each joint can be 0 and 3 respectively. The total minimum and maximum clinical disease activity score for each mouse can be 0 and 12 respectively. The number of mice per cohort was PBS n = 5, TT32 (17.46 nmol) n = 6, TT32 (6.55 nmol) n = 5, sCR1–10 (17.46) n = 5, and sCR1–10 (6.55 nmol) n = 5.

cascade in the pathogenesis of RA because T cells within the joints recognize fragments of autoantigens presented by dendritic cells (Benoist and Mathis, 2000). It has also been shown that B cells produce immunoglobulins directed against joint specific structures. Mice treated with a high dose of (2 mg) TT32, three times per week, had significantly ($p < 0.05$) reduced the levels of IgG2a in CIA (Fig. 12B), indicating that it impaired the antibody response in mice with arthritis consistent with the decrease in CDA and C3 deposition in the joints by TT32. Therefore, one can speculate that TT32 may have an effect on antigen presentation by B cells in arthritic mice. This decrease in the levels of IgG2 in mice treated with TT32 could be due to the fact that CR1 and CR2 play important roles in the antibody formation. CR1 and CR2 KO mice have defective B cell antigen presentation and antibody generation. CR1 and CR2 are receptors for C3-derived activation fragments (C3b, iC3b, C3d, C3dg) and targeting these C3b and C3d fragments was the main focus of current study. These are the fragments of C3 found abundantly in the joint microenvironment during acute inflammation. Of these, CR2 has been identified as a crucial link between innate and adaptive immunity by bridging the complement system to B cell-mediated humoral immune responses. Furthermore, the phenotype of *CR2*$^{-/-}$ mice deficient both in CR2/CR1 is characterized by multiple immunological alterations, including an altered natural antibody repertoire and impaired B cell responses to antigen exposure. CR2 deficiency has also been shown to protect mice from CIA (Kuhn et al.,

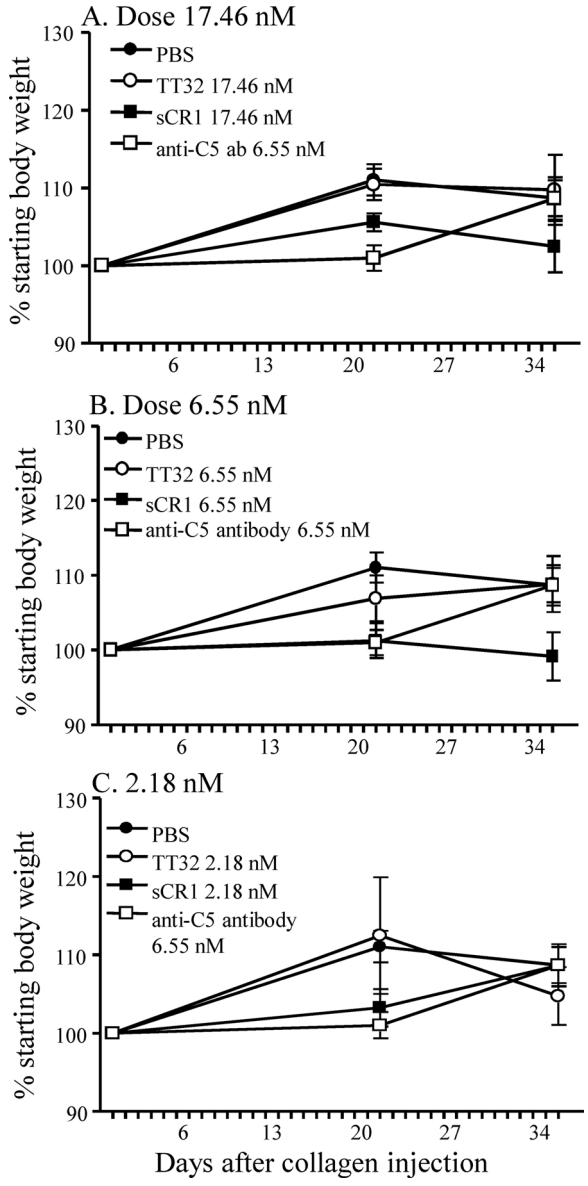


Fig. 11. No major change in the body weights of mice with CIA before and after treating with equivalent molar doses of human TT32 and sCR1–10. All mice were weighed prior to the induction of CIA, at day 1, after the induction of disease, at day 21 and after the termination of experiment, at day 35. Data are presented as percent (%) starting body weight change. **A.** Mice treated TT32 (17.46 nm) and sCR1–10 (17.46 nm). **B.** Mice treated TT32 (6.55 nm) and sCR1–10 (6.55 nm). **C.** Mice treated TT32 (2.18 nm) and sCR1–10 (2.18 nm). Mice were also treated with PBS and anti-C5a mAb (6.55 nm). The data represent the mean ± SEM for each group (n = 5) except for TT32 6.55 nmol (n = 6).

2008). We found that prophylactic administration of antibody 4B2, a mAb that binds to mouse CR2 and blocks the CR2-C3d interaction, substantially reduced levels of pathogenic IgG2a antibodies in CIA. Suppression of the IgG2a antibody response to bovine type II collagen (inducing immunogen) and to endogenous type II collagen was maintained for 6 weeks in that study (Kulik et al., 2015).

Previous studies have shown the correlation between CIA in mice and IgG autoantibody response to type CII (Williams et al., 1998). IgG2a autoantibodies are predominately present in arthritis (Watson and Townes, 1985). However, mice treated with only one 2 mg dose of TT32 per week had a similar level of disease activity as did mice dosed three times per week but did not show a statistically significant

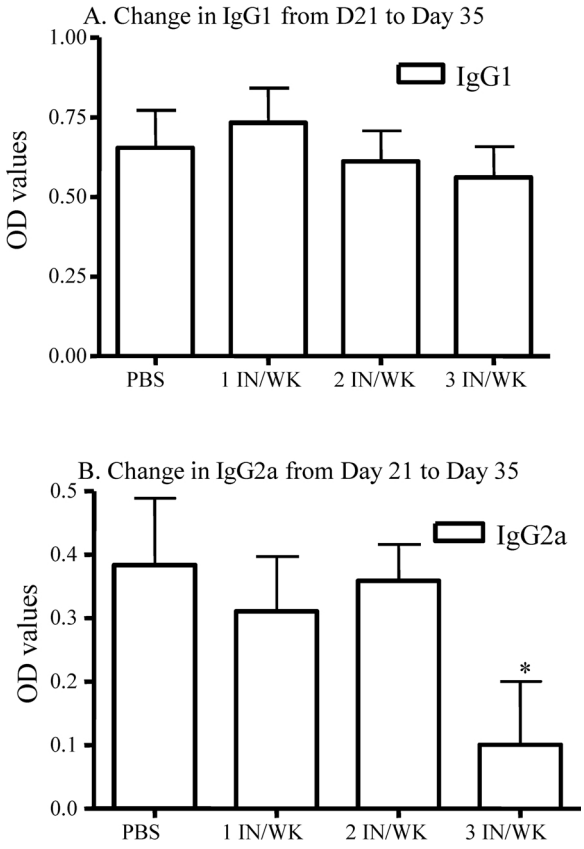


Fig. 12. Anti-CII responses in mice with CIA treated three times weekly with TT32. Following induction of CIA, mice were dosed either once, twice or thrice per week with a constant dose of TT32 (2 mg/mouse/i.p.) Mice injected with PBS alone served as a positive control. Sera from all mice were analyzed for collagen-specific IgG1 and IgG2a responses at day 0, day 21 and at day 35 as described in Material and Methods. All data are represented a change from day 21 to day 35 as mean OD + SEM. **A.** Change in the levels of IgG1. **B.** Change in the levels of IgG2a. *p < 0.05 compared with mice injected with PBS alone using ANOVA. IN/WK = number of injections per week.

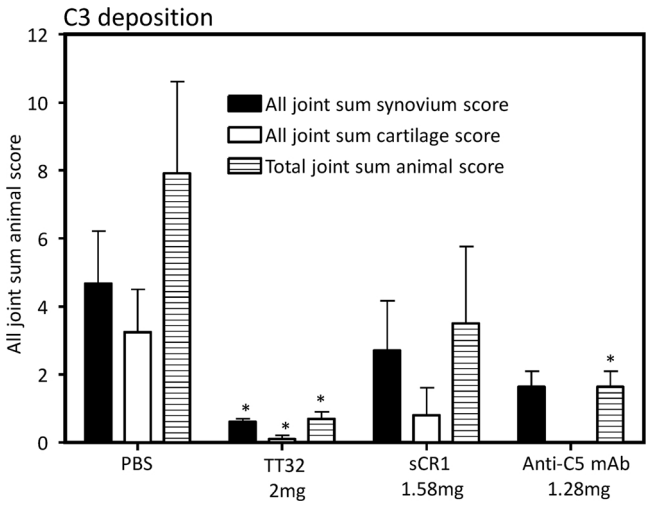


Fig. 13. C3 deposition, at day 35, from the DBA/Lac1j mice with CIA treated with TT32. All joints from the CIA mice treated with TT32 (2 mg), sCR1 (1.28 mg) and anti-C5 mAb (1.28 mg) were examined for C3 deposition in the synovium and on the cartilage surface staining as mentioned in the Materials and Methods. Data shown are from the synovium, cartilage and all joint sum animal scores. C3 deposition from all joint sum animal scores were shown as Mean ± SE. Total n = 6 for TT32, n = 5 for sCR1 and n = 4 for anti-C5 mAb. *p < 0.05 in comparison to the WT mice treated with PBS alone.

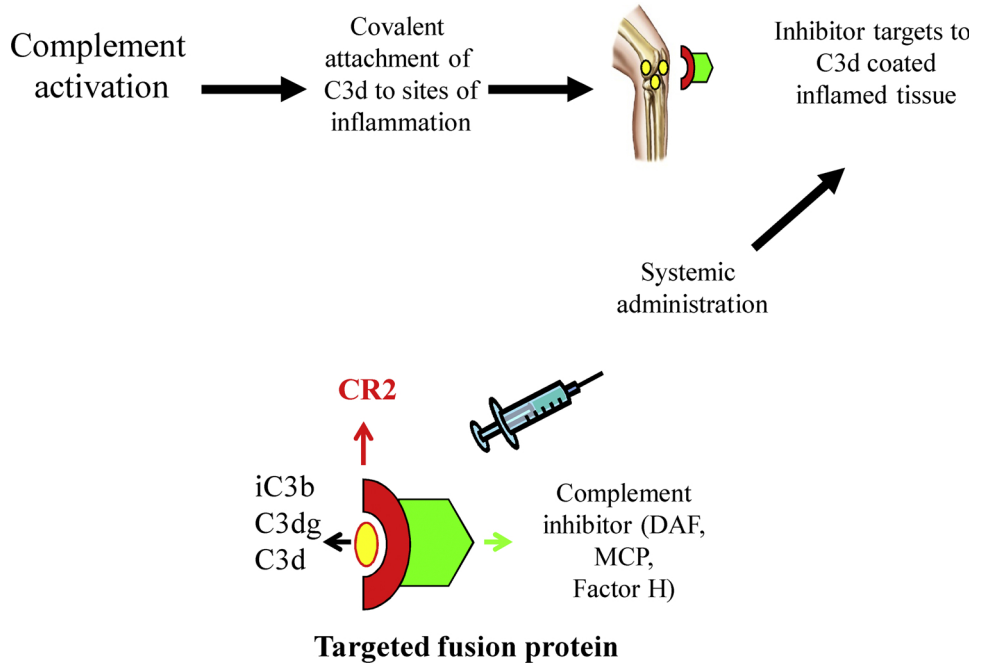


Fig. 14. Model showing the split components of C3 which might be the targets of TT32 fusion protein in the knee joint during inflammation and its therapeutic use in a clinical setting.

reduction in antibody levels. Although the circulating half-life of TT32 is short, the efficacy of TT32 in these models of inflammatory arthritis confirms its ability to inhibit both the AP and CP at the level of C3 and further extends the utility of targeting to areas of complement deposition by CR2. We plan to compare this broad spectrum complement inhibitor against pathway-specific inhibitors to better understand how individual complement components contribute to pathology. We hope to apply this knowledge to the development of inhibitors that are tailored for specific diseases.

5. Concluding remarks

These studies were initiated to evaluate the *in vitro* and *in vivo* activity of a human CR2-linked inhibitor, designated TT32, which was designed to block all three complement pathways at the C3 and C5 convertase levels in a tissue-fixed C3d-targeted manner. The results demonstrated the anticipated effects in that both the CP and AP were inhibited in human and mouse serum. Furthermore the results demonstrated that the potency of TT32 is maximized by the inclusion of the CR2 targeting moiety. *in vivo* experiments using the CAIA and CIA models confirmed the expected ability to ameliorate in a CR2-dependent manner inflammation and tissue injury in a complement-mediated disease model. Notably, TT32 is the most potent CR2-linked inhibitor tested in our studies, as it demonstrated activity against both the CP and AP as well as significantly more potent *in vitro* inhibitory effects than TT30 (specific for AP). These studies advance the field of tissue-targeted inhibitors by demonstrating the human CR1 activities can be built into a CR2-targeted inhibitor.

Authorship contributions

V.M.H. conceived the strategy, supervised the project and revised the manuscript. M.F.H. designed and carried out the inhibition studies, analyzed the data and prepared part of the manuscript. K.J., R.A., E.O and J.H prepared and characterized proteins. K.J. and J.F. also edited certain parts of the manuscript. F.S. conducted the experiments with the inhibitory/non inhibitory monoclonal antibodies including data analysis. M.F.H re-plotted some data for consistency. T. P. and Y. W. performed the pharmacokinetics studies. K.J assessed the PK samples and performed some inhibition studies. N.K.B. and G.M. performed *in vivo* CAIA and CIA studies, and N.K.B. analyzed CAIA and CIA data and wrote the initial, final and revised drafts of the manuscript. M.S. designed the expression constructs of TT32, oversaw the laboratory execution of the *in vitro* studies and wrote part of the manuscript. S.K. and A.S.L. reviewed the manuscript in-depth.

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