C5a Receptor (CD88) Blockade Protects against MPO-ANCA GN


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ABSTRACT

Necrotizing and crescentic GN (NCGN) with a paucity of glomerular immunoglobulin deposits is associated with ANCA. The most common ANCA target antigens are myeloperoxidase (MPO) and proteinase 3. In a manner that requires activation of the alternative complement pathway, passive transfer of antibodies to mouse MPO (anti-MPO) induces a mouse model of ANCA NCGN that closely mimics human disease. Here, we confirm the importance of C5aR/CD88 in the mediation of anti-MPO-induced NCGN and report that C6 is not required. We further demonstrate that deficiency of C5a-like receptor (C5L2) has the reverse effect of C5aR/CD88 deficiency and results in more severe disease, indicating that C5aR/CD88 engagement enhances inflammation and C5L2 engagement suppresses inflammation. Oral administration of CCX168, a small molecule antagonist of human C5aR/CD88, ameliorated anti-MPO-induced NCGN in mice expressing human C5aR/CD88. These observations suggest that blockade of C5aR/CD88 might have therapeutic benefit in patients with ANCA-associated vasculitis and GN.


Necrotizing and crescentic GN (NCGN) and vasculitis are associated with ANCA.1,2 ANCA-antineutrophil cytoplasmic antibodies (ANCA) are specific for myeloperoxidase (MPO) and proteinase 3 (PR3).1 Experimental data indicate that the pathogenesis of ANCA-associated vasculitis (AAV) involves activation of neutrophils by ANCA.1,2 Injection of ANCA antibodies into mice causes NCGN and vasculitis, closely mimicking human AAV.3 Alternative complement pathway activation is pivotal in the pathogenesis of ANCA NCGN in mice.4–6 The relevance of alternative complement pathway activation to human AAV is supported by immunohistochemical demonstration of alternative complement pathway components at sites of AAV7,8 and by correlation of plasma alternative complement pathway activation fragments with AAV disease activity.9

The complement anaphylatoxin C5a is a potent inflammatory mediator.10,11 The alternative classic and lectin pathways converge at the activation of C5, releasing C5a and C5b. C5a is a powerful chemoattractant for neutrophils, and ligation by C5a of C5aR/CD88 activates neutrophils. Blockade of C5a or C5a receptor (C5aR/CD88) ameliorates alternative complement pathway in mice.5,6 ANCA-activated neutrophils activate the alternative complement pathway.4,6,12 Neutrophil priming results in increased availability of ANCA antigens at the surface where they interact with ANCA to activate neutrophils. Human neutrophils activated by human ANCA release factors that activate the alternative complement pathway.4,6,12 In turn, C5a primes neutrophils and increase ANCA antigen expression.6,12 Cleavage of C5 also releases C5b, which joins with C6 to initiate the membrane attack complex (MAC).11

Here we confirm the importance of C5aR/CD88 in mediating anti-MPO NCGN and report that C6 is not required. We also demonstrate that deficiency of another receptor for C5a, C5L2 (C5a-like receptor 2),10 results in more severe disease. This is in accord with earlier studies that have shown an anti-inflammatory effect of C5L2 engagement.10,13,14

Therapeutic implications were investigated using CCX168, an antagonist of ANCA antigens at the surface where they interact with ANCA to activate neutrophils. Human neutrophils activated by human ANCA release factors that activate the alternative complement pathway.4,6,12 In turn, C5a primes neutrophils and increase ANCA antigen expression.6,12 Cleavage of C5 also releases C5b, which joins with C6 to initiate the membrane attack complex (MAC).11

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of human C5aR/CD88 that is undergoing phase 2 evaluation in patients with AAV (EU Clinical Trials Register ID: EUCTR2011–001222–15–GB). Oral administration of CCX168 to humanized mice with knocked-in human C5aR/CD88 ameliorated anti-MPO NCGN.

RESULTS

C5aR/CD88 Deficiency Ameliorates, C5L2 Deficiency Exacerbates, and C6 Deficiency Has No Effect on Anti-MPO–Induced NCGN

Injection of 50 μg/g mouse antimumose MPO IgG into wild-type (WT) B6 mice resulted in NCGN (Figure 1A) in all mice (n=7) with an average of 8.1% crescents (Figure 1B). B6 mice with knocked-out C5aR/CD88 were protected (P=0.0359), with only 1 of 6 mice developing 1% crescents (Figure 1B). In contrast, B6 mice with knockout of C5L2 (C5a-like receptor 2) had more severe disease (P=0.0035), with 18% crescents (Figure 1B). These observations support a proinflammatory function for C5aR/CD88 and an anti-inflammatory inhibitory function for C5L2, as previously reported.10,13

C5 activation is required to induce anti-MPO NCGN.5,6 C5 activation generates C5a and C5b. C5a mediates inflammation by attracting and activating neutrophils, whereas C5b joins with C6 to initiate MAC assembly.11 To investigate the pathogenic role of C6, NCGN induction by anti-MPO IgG in WT C3H/HeJ mice was compared with that in C6−/− C3H/HeJ mice. After injection of anti-MPO IgG, all WT C3H/HeJ mice and all C6−/− C3H/HeJ mice developed NCGN, with an average of 5.2% crescents in WT mice and 4.4% in C6−/− mice (P=0.63) (Figure 1C). Thus, C6 and MAC are not important for the pathogenesis of anti-MPO NCGN.

Mouse Anti-MPO IgG Induces NCGN in Mice with Knocked-In Human C5aR/CD88 and Knocked-Out Mouse C5aR/CD88

The role for C5aR/CD88 in anti-MPO NCGN in mice has implications for C5aR/CD88 blockade as therapy for AAV. We tested the ability of a small molecule antagonist of human C5aR/CD88 to ameliorate anti-MPO NCGN in mice with the murine C5a receptor knocked out and replaced with the human C5a receptor (hC5aR mice). Injection of mouse anti-MPO into hC5aR mice induced NCGN with an average of 30% crescents, which was similar to induction of disease by anti-MPO in WT littersmates that developed 39% crescents (P=0.61) (Figure 2A). The hC5aR mice have a mixed genetic background of 129S6 and B6. The higher percentage of crescents in these mice compared with that in B6 mice is consistent with our observation that 129S6 mice are genetically predisposed to more severe disease and that crosses between 129S6 and B6 mice have intermediate severity.15

Mouse Leukocytes with Knocked-In hC5aR/CD88 and Knocked-Out Mouse C5aR/CD88 Respond to an Antagonist of hC5aR/CD88

Flow cytometry demonstrated hC5aR but not mC5aR on hC5aR knock-in mice (Figure 3A). CCX168 markedly retarded chemotaxis of leukocytes from hC5aR mice (Figures 3B). Oral pretreatment of hC5aR mice with a single dose of CCX168 inhibited the exudation of peritoneal neutrophils in response to thioglycollate (Figure 3C) as well as transient depletion of blood neutrophils caused by intravenous C5a administration (Figure 3, E and F), thus documenting an inhibitory effect of CCX168 on leukocyte function in hC5aR mice.

A Small Molecule Inhibitor of hC5aR/CD88 (CCX168) in Mice with hC5aR/CD88 Ameliorates Anti-MPO–Induced NCGN

Oral CCX168, 30 mg/kg daily, reduced the severity of anti-MPO NCGN in hC5aR mice. Glomerular crescents were reduced from 30.4% to 3.3% with CCX168 (P<0.0001) (Figure 2A). Urine hematuria, proteinuria, and leukocyturia were reduced in mice receiving CCX168, 30 mg/kg per day (Figure 2B).

The protection by CCX168 resulted in reduced crescents (P<0.001) (Figure 2C) and necrosis (P<0.001). In control mice versus CCX168-treated mice, there were more glomeruli with neutrophils (33.2% versus 12.0%; P=0.007) and more neutrophils per glomerulus (1.2 versus 0.2; P=0.003). The amelioration of anti-MPO NCGN by CCX168 was dose dependent (Figure 2C). Thus, an antagonist of human C5aR/CD88 effectively ameliorates anti-MPO induced NCGN in mice with hC5aR/CD88.

DISCUSSION

Acute inflammatory lesions of AAV are extremely destructive and characterized histologically by influx or neutrophils, leukocytoclasia, and necrosis.16,17 These lesions of AAV indicate that the pathogenic inflammatory mechanism involves intense recruitment and activation of leukocytes, especially neutrophils.

The complement system components inflammation that has been initiated by many inflammatory stimuli. The alternative complement pathway is particularly effective at augmenting and sustaining acute inflammation because it has a self-fueling amplification loop.11

Observations in human AAV and in experimental animal models of AAV support an important role for the alternative complement pathway. The lesions of AAV are characterized by a paucity of immunoglobulin and complement, especially compared with typical immune complex or anti–glomerular basement membrane–mediated GN and vasculitis; however, low-intensity deposition of complement is common, especially at focal sites of inflammation and necrosis.7,8 Xing et al. detected factor B, properdin, MAC, and C3d in glomeruli and small blood vessels with active AAV, which suggested alternative pathway activation.7 Gigante et al. also detected complement components in AAV lesions and observed that the extent of lesion C3c correlated with poor renal
Figure 1. C5aR (CD88) deficiency ameliorates, C5L2 deficiency enhances and C6 deficiency has no effect on anti-MPO–induced GN. WT B6, C5L2−/− B6, C5aR/CD88−/− B6, WT C3H/HeJ, and C6−/− C3H/HeJ were injected intravenously with 50 µg/g body weight mouse anti-mouse MPO IgG and euthanized on day 6. Glomerular crescents were calculated as percentage of glomeruli with crescents by counting all glomeruli in cross-sections of both kidneys, which averaged approximately 80 per cross-section. (A) In contrast to histologically unremarkable glomeruli (upper left, C5aR−/− mouse), glomeruli with crescents had increased extracapillary cells often with adjacent segmental fibrinoid necrosis (upper right, C5L2−/− mouse; lower left, C6−/− mouse; lower right, hC5aR mouse). (B) All WT B6 mice developed glomerular crescents with an average of 8.1% of glomeruli with crescents, B6 mice with knocked-out C5aR/CD88 were protected from induction of GN by anti-MPO IgG, with only 1 of 6 mice developing rare crescents, and B6 mice with knockout of C5L2 had more severe disease, with an average of 18% crescents per mouse (P=0.0035). (C) All WT C3H/HeJ mice and all five C6 knock out C3H/HeJ mice developed NCGN, with an average of 5.2% of glomeruli with crescents in WT mice and 4.4% in C6−/− mice (P=0.63). Thus, the absence of C6 did not influence pathogenicity of anti-MPO. Magnification, ×400, periodic acid-Schiff stain.
outcome. In patients with AAV, Gou et al. reported increased plasma levels of C3a, C5a, soluble C5b-9, and Bb in patients with active disease but not remission. The plasma Bb correlated with percentage of crescents. Thus, data from tissue specimens and plasma samples support a role for alternative complement pathway activation in AAV.

Animal models that closely mimic human AAV are induced by circulating anti-MPO in mice. The alternative complement pathway is required for disease induction by anti-MPO. Blockade of C5aR or C5L2 deficiency had the opposite effect of C5aR/CD88 deficiency, indicating that C5aR/CD88 engagement enhances inflammation whereas C5L2 engages suppresses inflammation, which has been proposed before.

C5 activation generates not only C5a but also C5b, which complexes with C6 to initiate the membrane attack complex (MAC) (C5b-9d). We examined the importance of the C6 in murine anti-MPO NCGN using C6-deficient mice.

Figure 2. C5aR/CD88 small molecule antagonist CCX168 ameliorates anti-MPO–induced GN. Daily administration of 30 mg/kg CCX168, an orally active small molecule antagonist of human C5aR, markedly reduced the severity of anti-MPO–induced NCGN in hC5aR knock-in mice (7 days after anti-MPO injection). (A) Glomerular crescent formation induced by anti-MPO was reduced from 30.4% with vehicle alone to 3.3% with CCX168 (P<0.0001). (B) Urine hematuria, proteinuria, and leukocyturia were reduced in mice receiving CCX168 (dipstick scale, 0–4+). (C) Glomerular crescent and necrosis formation was reduced in animals receiving CCX168. Data were analyzed by Mann-Whitney t test; **P<0.01 and ***P<0.001. Data are presented as the median with SEM. BID, twice daily; QD, daily.
and observed no effect. This C6-deficient strain has been used effectively in other mouse models in which the C6 deficiency ameliorated MAC-mediated injury.18–20 MAC may be present in lesions but is not required to induce injury.

The amelioration of anti-MPO NCGN in mice with humanized C5aR/CD88 not only provides additional support for the important pathogenic role for complement activation in AAV but also supports the possibility that therapy directed at preventing or reducing complement activation might be beneficial in patients with AAV.

CONCISE METHODS

Mice

WT C57B6/J (B6) and C3H/HeJ mice were purchased from Mutant Mouse Regional Resource Centers (Columbia, MO). C5aR−/−, C5L2−/−, and litter mate WT control mice were provided by Dr. Craig Gerard (Harvard Medical School, Boston, MA). MPO−/− mice were maintained by the University of North Carolina Division of Laboratory Animal Medicine. MPO−/− mice (8–10 weeks old) were used for immunization and as donors of anti-MPO antibodies using previously published methods.5 Animal care and animal experiments were conducted in
accordance with the Animal Care Committee at the University of North Carolina, Chapel Hill, and National Institutes of Health Guide for Care and Use of Laboratory Animals in Research.

Humanization of Mice with Human C5aR
Standard homologous recombination techniques were used to create mice with the murine C5a receptor replaced with the human C5a receptor. These mice had a mixed genetic background of 129S6 and C57BL/6. In addition to standard confirmation by genotyping, the effectiveness of replacement of the mC5aR with the hC5aR was tested by determining leucocyte expression of mC5aR versus hC5aR on peripheral blood leukocytes by flow cytometry, and by measuring CCX168 suppression of human C5a-induced chemotaxis of thioglycollate-induced peritoneal leukocytes from mC5aR versus hC5aR mice. CCX168 was prepared by the Medicinal Chemistry Department at ChemoCentryx and formulated in polyethylene glycol 400/Solutol (70/30). Response to human C5a of hC5aR knock-in mouse leukocytes was tested in vitro using a previously described chemotaxis assay. In brief, migration of cells from the upper to the lower ChemoTX chamber (NeuroProbe, Gaithersburg, MD) in response to different concentrations of human C5a was determined by adding CyQUANT solution (Invitrogen) to each lower chamber and measuring the intensity of fluorescence (Migration Signal) of the DNA-binding fluorescent CyQUANT after 120 minutes, which is a relative measure of cell numbers. In vitro, human C5aR responds equally well to murine C5a and human C5a (data not shown), which is in accord with previously reported results. The cross-reactivity of CCX168 has at least four orders of magnitude less reactivity versus C5aR (data not shown). According to use of a previously described method, the effect on in vivo chemotaxis of oral pretreatment 2 hours before intraperitoneal thioglycollate injection with vehicle or a single dose of 30 mg/kg of CCX168 on cell count in peritoneal lavage was measured 24 hours after intraperitoneal injection of thioglycollate. CCX168 effects on C5a-induced leukopenia was studied in hC5aR knock-in mice 1 hour after oral administration of CCX168 by comparing leucocyte counts in blood drawn 1 minute before and 1 minute after intravenous administration of C5a (20 μg/kg).

Preparation of Pathogenic Mouse Antimurine MPO IgG
Purification of native mouse MPO and immunization of MPO−/− mice were performed as previously described. Briefly, 8- to 10-week-old MPO−/− mice were immunized intraperitoneally with 20 μg of purified murine MPO in complete Freund adjuvant and boosted twice with 10 μg MPO in incomplete Freund adjuvant. Development of anti-MPO antibodies was monitored by ELISA. Anti-MPO IgG was isolated from serum of MPO−/− mice immunized with MPO by 50% ammonium sulfate precipitation and protein G affinity chromatography. Purity of antibodies was confirmed by SDS-PAGE electrophoresis, and the purified IgG was diazylzed against PBS and sterile-filtered. The protein concentrations were determined by Coomassie protein assay (Pierce, Rockford, IL).

Induction of Experimental GN with Anti-MPO IgG and Amelioration by C5aR Blockade
Induction of experimental necrotizing and crescentic glomerulonephritis (NCGN) with anti-MPO IgG in mice was performed as previously described. WT C3H/HeJ (n=5), C6−/−/C3H/HeJ (n=5), wild type B6 (n=7), B6 C5aR−/− (n=6), B6 C5L2−/− (n=7) and hC5aR mice were injected intravenously with 50 μg/g body weight mouse anti-mouse MPO IgG and placed in metabolic cages for 12 hours on day 3 to collect urine for analysis, and sacrificed on day 6. Humanized hC5aR mice received varying oral doses of CCX168 C5aR antagonist or vehicle starting on day 1 and ending on day 6. An oral dose of 30 mg/kg b.i.d. CCX168 was selected for the initial proof of concept because preliminary studies showed that this dose is well tolerated in mice and because the resultant plasma concentration of CCX168 was suitable for efficacy studies, peaking one hour after dosing and retaining approximately 140 nM CCX168 in the plasma for 24 hours, which is sufficient to fully inhibit C5aR. Additional dose-response evaluations at 0.1 mg/kg four times daily, 1 mg/kg four times daily, and 4 mg/kg twice daily were used.

Urine was tested by dipstick for hematuria, proteinuria, and leukocyturia (Roche Diagnostics Corp., Indianapolis, IN). Kidney specimens were fixed in 10% buffered formalin and prepared for light microscopy using hematoxylin–eosin and periodic acid–Schiff staining. Glomerular crescents and necrosis were calculated as percentage of glomeruli with crescents or necrosis by counting all glomeruli in cross-sections of both kidneys, which averaged approximately 80 per cross-section. To evaluate neutrophil infiltration, immunohistochemical staining was carried out on paraffin sections using a biotin-labeled rat monoclonal antimouse neutrophil antibody (Cedarlane Laboratories Ltd., Burlington, Ontario, Canada), with detection with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) and a DAKO Liquid DAB-chromogen system (Dako, Carpinteria, CA). Results were expressed as the percentage of glomeruli with neutrophils and the number of neutrophils per glomerulus.

Statistical Analysis
All data are presented as mean ± SD. The data were analyzed using a t test and one-way ANOVA with Bonferroni multiple comparison post-test. P<0.05 was considered to indicate a statistically significant difference. All analyses were done using SAS software (version 9.3; SAS Institute, Cary, NC).

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DISCLOSURES

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REFERENCES


See related editorial, "With Complements from ANCA Mice," on pages 207–209.