Role of C3a Receptors, C5a Receptors, and Complement Protein C6 Deficiency in Collagen Antibody-Induced Arthritis in Mice


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The complement system, especially the alternative pathway, plays essential roles in the induction of injury in collagen Ab-induced arthritis (CAIA) in mice. The goal of the current study was to directly compare the roles of receptors for C3a and C5a, as well as the membrane attack complex, as effector mechanisms in the pathogenesis of CAIA. Clinical disease activity in C3aR−/−, C5aR−/−, and C6-deficient (C6-def) mice was decreased by 52, 94, and 65%, respectively, as compared with wild-type mice. Decreases in downstream of all three effectors that promote additional IgG deposition and C3 activation in the joint. led to differential deficiencies in effector mechanisms, decreased proximal joint IgG and C3 deposition was common to all three susceptibility to CAIA, with clinical effects most pronounced in C5aR-deficient mice. Although the absence of C3aR, C5aR, or C6 cytokine. Our findings show that absent C3aR-, C5aR-, or membrane attack complex-initiated effector mechanisms each decrease C3aR

Rheumatoid arthritis is an inflammatory autoimmune arthritis for which pathogenesis is complex (1, 2). To better understand the proinflammatory pathways involved in this disease, mouse models of inflammatory arthritis such as collagen-induced arthritis, anti-GPI Ab-induced arthritis (anti-GPI Ab), and collagen Ab-induced arthritis (CAIA) have been devised and studied extensively. Our laboratory, along with others, has shown a significant and necessary role for the alternative pathway (AP) of complement in the pathogenesis of inflammatory arthritis in mice (3–9).

Complement is a potent effector pathway of innate immunity that accomplishes its biologic roles in inflammation by using several key proinflammatory and immunomodulatory mechanisms. C3 and C5 are central pillars of this system. For example, all three activation pathways of the complement system converge to form a C3 convertase, or activating enzyme, which cleaves C3 into the C3a and C3b fragments. The generation of C3b leads to the formation of another enzyme, the C5 convertase, which then cleaves C5 into the C5a and C5b fragments. C3a and C5a fragments are the most potent proinflammatory anaphylatoxins generated during complement pathway activation. These anaphylatoxins recruit and/or activate monocytes/macrophages and neutrophils, which themselves are involved in a myriad of pathologic states including those of autoimmune diseases, inflammatory reactions, allergic reactions, asthma, and cancer (10–14). In addition to anaphylatoxins, through the formation of the membrane attack complex (MAC) the complement system eliminates pathogens through lysis and causes local tissue injury by the initiation of proinflammatory signaling in cells.

C3a and C5a induce their biological actions through two specific receptors designated the C3aR and C5aR (CD88), respectively (15, 16). C5L2 (GPR77) is another nonsignaling receptor for C5a (17–19), which likely acts as a decoy receptor and a negative modulator of C5a-induced responses (20). Both major receptors for C3a and C5a belong to a family of transmembrane G protein-coupled receptors, but C5L2 is not coupled to the G proteins (17, 21). Many different cell types express receptors for C3a and C5a. These include cells of myeloid origin (22), nonmyeloid origin (23), dendritic cells (24), monocyte/macrophages (25), and neutrophils (26). Resting mast cells express C5aR below threshold levels, but stimulation with PMA or ionomycin results in increases in C5aR expression as well as in slight increases in C3aR receptors (27).
Following C5b generation, C6 interacts with C5b on the cell surface to begin formation of the MAC (C5b, C6, C7, C8α, C8β, C8γ, and C9) that can insert into any cell membrane (28). Deficiency of any of the aforementioned proteins of the MAC would block the formation of the lytic complex in the membrane of cells. If present at high enough levels, the MAC causes lysis of cells; perhaps more importantly, when present in sublytic concentrations, the MAC also transduces proinflammatory cell activation signals (29). The MAC also plays a role in the cell cycle and apoptosis (30, 31) and has been shown to be present in rheumatoid arthritis tissue, suggesting a role in the pathogenesis of arthritis (32).

Serum-induced arthritis in K/BxN mice is mediated by anti-GPI-Igs (33), and the development of disease requires both C5aR and FcγRIII receptors (7). This study also showed that disease was dependent on the AP of complement. In contrast, C6-deficient (C6-def) mice on a C3H/He background were fully susceptible to anti-GPI Ab-induced arthritis (37), suggesting a lack of a role for the MAC. In contrast, there was a 60% decrease in monosodium urate monohydrate crystal-induced arthritis in New Zealand White rabbits deficient in C6 (34). This study reported infiltration of mononuclear leukocytes in the synovial tissue in all animals, but substantial infiltration of neutrophils was also seen in C6-def rabbits in response to monosodium urate monohydrate crystals. In addition, in a mouse model of chondrocalcinosis (CNV) (i.e., laser-induced CNV, which involves inflammation), anti-C6 mouse polyclonal Ab inhibited the formation of the MAC and resulted in amelioration of CNV (35). Anti-C6 F(ab)2 also reduced experimental autoimmune myocarditis gravis disease passively induced in rats (36), showing the dependency of this disease on MAC formation.

Despite this body of work on the role of individual effector pathways, no prior study has directly compared the relative roles of these three major effector pathways in CAIA in C57BL/6 mice. Our hypothesis was that the interactions between C3a–C3aR and C5α–C5αR and a deficiency of MAC formation would result in differential effector functions and thus lead to different disease phenotypes in C3α-def, C5α-def, and C6-def mice. Although we found that each effector demonstrated unique characteristics with regard to changes in downstream proinflammatory effects in their absence, there were similar ameliorative effects on the disease course itself. One unexpected feature shared by all effector-deficient mice was a decrease in the ameliorative effects on the disease course itself. One unexpected proinflammatory effects in their absence, there were similar C6-def mice. Although we found that each effector demonstrated

### Materials and Methods

#### Mice

Ten- to 12-week-old homozygous C3−/−, C3αR−/−, C5αR−/−, and C6-def C57BL/6 male and female mice were used for this study of CAIA, C3αR−/− and C5αR−/− mice were obtained from Dr. Rick Wessel (University of Texas, Houston, TX), and C6-def mice were obtained from Dr. Tod Merkel (Center for Biologies Evaluation and Research, Food and Drug Administration). Because C57BL/6 mice naturally lack complement protein C6 and are not gene-targeted mice, in the current studies, these mice have been designated as C6-deficient (C6-def). These C6-def C57BL/6 mice have been derived from a C6-def C3H/He mouse strain, as narrated in detail below. Sera from C1q−/−, C3−/−, C4−/−, Bf−/−, and Df−/− mice backcrossed to at least F10, as well as C5-deficient NOD mice, were used as negative controls in ELISA assays as described. Age- and sex-matched C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used as wild-type (WT) controls. Genotypes of deficient strains were confirmed by deletion-specific DNA PCR analysis prior to use of the animals. The studies were performed in four different cohorts with the following total mice studied: WT, n = 25; C3−/−, n = 4; C3αR−/−, n = 13; C5αR−/−, n = 11; and C6-def, n = 8. All animals were kept in a barrier animal facility with a climate-controlled environment providing 12-h light/dark cycles. Filter-top cages were used with three 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.

#### Derivation of C57BL/6 C6-def mice

C6-def C3H/He mice were derived from a Peru-Peacock strain of mice that lacked functional C6 (37). The molecular basis of the deficiency was determined to be due to the presence of several base substitutions in the C6 allele relative to the WT allele (38). These base substitutions result in the presence of restriction enzyme site BstNI in the WT allele that is absent in the C6-def allele and the presence of restriction enzyme site AlwNI in the C6-def allele that is absent in the WT allele. This allowed for unambiguous identification of heterozygous mice, mice homozygous for the C6-def allele, and mice homozygous for the WT allele. Genomic DNA was isolated from whole blood from individual mice using the MoBio UltraClean DNA BloodSpin Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer’s instructions. DNA amplification was performed using primers C6DE-F1 (5′-GAGCCCTTGGACCCTGCTCATTGTCCA-3′) and C6DE-R1 (5′-GGACCTGCGCTACAGTTCTCA-3′) using Roche PCR Master Mix (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. The resulting PCR product was purified and digested with restriction enzymes BstNI and AlwNI. The restriction patterns for each digestion were analyzed to determine whether the WT allele, the C6 allele, or both alleles were present. To transfer the C6-def allele to the C57BL/6 background, C6-def C3H/He mice were backcrossed eight times to C57BL/6 mice. Following each backcross, offspring homozygous for the C6-deficient allele were identified using the PCR/restriction enzyme screen described above and crossed with WT C57BL/6 mice. Following the eighth backcross, offspring homozygous for the C6-deficient allele were selected and used to set up homozygous breeding pairs. The offspring of these breeding pairs were confirmed to be homozygous for the C6-def allele using the PCR/restriction enzyme screen described above and determined to be deficient for complement-mediated lysis in an RBC assay and to lack C6 protein by Western blot (data not shown). A coagulation defect, characterized by decreased platelet aggregation, was described in C6-def mice (29, 31). It is not known if a similar coagulation defect is present in the C6-def C57BL/6 mice used in this study.

#### Induction of CAIA

CAIA was induced in C3αR−/−, C5αR−/−, and C6-def, and WT mice by using a mixture of four mAb to bovine type II collagen (CII) (Arthrogen, Clarksburg, MD) suspended in sterile saline and injected via the tail vein on days 7 and 9 (39). After 14 days, arthritis was scored (40). The studies were performed in four separate experiments. The first experiment consisted of C3αR−/− (n = 5), C5αR−/− (n = 5), and WT (n = 6) mice; the second consisted of C3αR−/− (n = 3), C5αR−/− (n = 2), C6-def (n = 3), and WT (n = 4) mice; the third consisted of C3αR−/− (n = 5), C5αR−/− (n = 4), and WT (n = 4) mice; and the fourth consisted of C6-def (n = 5) and WT (n = 11) mice. For presentation of results, all genotype-identical mice were grouped together from each of the four experiments and included in the final analyses.

#### Examination for clinical disease activity

The prevalence of disease and severity of clinical disease activity (CDA) in all C3αR−/−, C5αR−/−, C6-def, and WT mice were determined every day by a trained laboratory person blinded to the experimental treatment group according to our previously published studies (35). The CDA score is based on a three-point scale per paw: 0, normal joint; 1, slight inflammation and redness; 2, severe erythema and swelling affecting the entire paw with inhibition of use; and 3, deformed paw or joint with ankylosis, joint rigidity, and loss of function. The total CDA score was based on all four paws with a maximum total of 12 for each mouse.

#### Histopathology and immunohistochemistry for C3 and IgG

Knee joints from both fore limbs and the right hind limb with knee joint, ankle and paw, from C3αR−/−, C5αR−/−, C6-def, and WT mice at day 10 following Arthrogen injection were fixed in 10% buffered formalin for 7 days. Toluidine-blue stain was used to assess histopathology scores for inflammation, pannus formation, cartilage, and bone damage, according to the published criteria (39) (Supplemental Material). Histology sections were...
cut (7 μm) and processed for C3 and IgG immunohistochemistry staining. Non-specific binding was blocked using a protein block serum-free solution (DakoCytomation). C3 was localized with a primary polyclonal goat anti-mouse C3 antiseraum (dilution 1:10,000) (ICN Pharmaceuticals) and detected by goat HRP polymer kit (BioCare Medical) (5). IgG was localized with a primary polyclonal goat anti-mouse Ab (dilution 1:200) (Vector Laboratories, Burlingame, CA) and detected by a goat HRP polymer kit, as mentioned above. Development of staining identifying the presence of tissue-fixed C3 and IgG was carried out using DAB plus solution substrate (DakoCytomation), which reacts with HRP and produces a brown color for positive staining. All slides for histopathology, C3, and IgG deposition were observed under light microscopy at a magnification of ×20 in a blinded fashion and scored according to published criteria (39). IgG deposition on the surface of cartilage in the knee joints of WT, C3aR−/−, C5aR−/−, and C6-def mice with disease was determined by using the following scale: 0, normal WT with no disease (i.e., background endogenous IgG staining); 1, minimum staining; 2, moderate staining; and 3, intense staining. The knee joints from RAG2−/− mice on C57BL/6 background with no disease were also used as negative controls.

Quantitative assessment of monocytes/macrophages and neutrophils in the knee joints

Histology sections from the knee joints of all C3aR−/−, C5aR−/−, C6-def, and WT mice with CAIA sacrificed at day 10 were also examined for the infiltration of monocytes/macrophages and neutrophils in the knee joints. To determine the percentages of monocytes/macrophages and neutrophils, sections were pretreated with proteinase K (0.05 mol/l; DakoCytomation) for 5 min for Ag retrieval followed by blocking with a serum-free protein block solution (no dilution) (DakoCytomation). Immunohistochemistry analysis for monocytes/macrophages was performed by staining sections with the anti-C3aR primary antibody (dilution 1:1000, Serotec) and a secondary rabbit anti-rat Ab (dilution 1:400) (DakoCytomation), as primary and secondary Ab, respectively. Rabbit Alkaline Phosphatase Polymer (no dilution) (BioCare Medical) was used as a tertiary Ab. Detection of monocytes/macrophages was performed using Vulcan Fast Red Chromogen (no dilution) (DakoCytomation). To determine the number of neutrophils, knee joint sections with a primary rat anti-mouse neutrophil Ab (dilution 1:2000) (Serotec) and a secondary rabbit anti-rat Ab (dilution 1:400) (DakoCytomation), followed by Rabbit Envision Polymer (no dilution) (HRP-conjugated) (DakoCytomation). Neutrophils were detected by using 3,3’-diaminobenzidine (no dilution) (DakoCytomation). The percentages of the monocytes/macrophages and neutrophils were calculated by marking the boundaries of synovium using Scan Scope XT (Aperio). The formula used to calculate such percentages was the total number of positively stained synovial area/total marked synovial area × 100. Although the areas of synovium were heterogeneous due to inflammation, all stained areas were included in assessing percentages of monocytes/macrophages and neutrophils.

Measurement of C3 deposition and C5a generation in vitro using sera from mice

Complement activity in sera from C3−/−, C3aR−/−, C5aR−/−, C6-def, NOD, and WT mice was assayed in vitro by measuring C3 deposition and C5a generation. Blood for these studies was collected by retro-orbital bleeding and allowed to clot for 30 min at 4˚C. After centrifugation at 3000 rpm for 15 min at 4˚C, sera were separated from the clot on ice and kept at −70˚C immediately after collection until further analysis. To avoid complement activation, sera were used only one time without being subjected to repeated cycles of freezing and thawing. Sera were discarded that showed any discoloration due to hemolysis. Frozen serum samples were thawed and 1/4 of the diluted sera were kept on ice prior to use. For analysis of all three pathways of complement activation, sera were serially diluted 2-fold from 1:10 in Ca2+/Mg2+/EGTA. These sera were then added to 96-well Costar ELISA plates precoated with anti-C3 mAb (Arcturus; 2.5 μg/ml) and incubated at 37˚C for 1 h. C3 deposition adherent to the plate and C5a generation in the supernatant were measured by ELISA, as previously described (4, 39).

Levels of complement components in the sera from complement-deficient and WT mice

Cytokine mRNA levels from the synovium of knee joints using laser capture microdissection

The left knee joint was surgically removed from mice with and without CAIA at day 10, the skin was removed, and the tissue was placed in liquid nitrogen. After removing extra muscle using a surgical blade, the knee joint was embedded for 5 min in Tissue-Tek O.C.T. Compound (Sakura Finetek). The histology sections were cut at 8 μm thickness (Leica Microsystems, Wetlar), mounted on Histostick slides (Stal Lab Medical Products, McKinney, TX). All slides were dehydrated for 30 s sequentially in 70, 95, and 100% ethanol and for 5 min in xylene (Arcturus Histogen, Carlsbad, CA). The slides were rehydrated in nuclease-free water (Arcturus Histogen) containing 50 μl RNase inhibitor (Sigma-Aldrich, St. Louis, MO) for 30 s to remove the O.C.T. compound. The slides were air-dried for 5 min at room temperature. The knee joint sections were then placed on the area around the identified synovium. The histo-pencil from the drawing tools was used to mark the boundaries of the synovium to be placed on the cap, and an infrared laser was fired. The infrared laser melted the polymer onto the cap, creating an adhesion of the cells to the polymer. The cap was moved to the quality control station to view the synovium. The cap with the synovium was placed in a tube with 50 μl RNA extraction buffer (Arcturus Histogen) and incubated for 30 min at 42˚C. RNA was isolated using the PicoPure RNA isolation kit (Arcturus Histogen) and then amplified (1.5–2.0 round amplification) using the RiboAmp Plus kit (Arcturus Histogen) by synthesizing cDNA strands from the isolated RNA with enhancers and transcripting it into amplified RNA (aRNA). The aRNA was purified using a column method supplied in the kit. RNA concentration was measured using a nanodrop. This aRNA was then used for quantitative RT-PCR (QRT-PCR) using specific primers for the mRNA levels of TNF-α, IFN-γ, IL-1β, and IL-10, as previously published (5, 39).

Statistical analyses

The p values were calculated using Student t test with the GraphPad Prism 4 statistical program (GraphPad). The data in graphs, histogram, and tables are shown as the mean ± SEM, with p < 0.05 considered significant using an unpaired two-tailed test. One-way ANOVA using Tukey’s multiple comparison test was also performed to further confirm the significant differences among WT, C3aR−/−, C5aR−/−, and C6-def mice for CDA, histology, C3 deposition/C5a generation, and QRT-PCR data. Pearson correlation was used to determine the correlation among CDA, neutrophils, macrophages, histology, and absolute serum complement levels. Preliminary analyses using a null hypothesis for w-statistics indicated that the data were usually normally distributed. Where data were not normally distributed, the Mann–Whitney U test was used.

Results

Clinical disease activity and prevalence of disease

CDA in all cohorts of mice was evaluated every day after the LPS injection on day 3. Mice injected with Arthrogen developed disease after day 3, and at day 10 the CDA in WT, C3aR−/−, and C5aR−/− mice was 10.8 ± 0.54, 5.2 ± 1.1, and 0.64 ± 0.20, respectively (Fig. 1A). These scores were significantly reduced by 52 and 94% in C3aR−/− and C5aR−/− mice, respectively, as compared with WT mice. The prevalence of disease at day 10 in WT, C3aR−/−, and C5aR−/− mice was 100, 85, and 55%, respectively (Fig. 1B). At day 10, the CDA in WT and C6-def mice was 9.3 ± 0.66 and 3.3 ± 0.41, respectively (Fig. 1C). The CDA in C6-def mice was significantly reduced by 65% as compared with WT mice. The prevalence of disease at day 10 both in WT and C6-def was 100% (Fig. 1D). C5aR−/− mice were more resistant to the development of CAIA as measured by CDA than C3aR−/− mice and C6-def mice (p < 0.01). No statistically significant (p = 0.18) difference was observed when comparing CDA between C3aR−/− and C6-def mice.

Differential patterns of disease development in C3aR−/− mice

We examined CDA in each mouse from days 3–10 in all four cohorts of mice. There were only scattered individual variations of CDA in C3aR−/− and C6−/− mice (Supplemental Fig. 1C, 1D). However, there was substantial variation in the individual CDA in
tested. The genotype of the mice was confirmed twice as C3aR−/− prior to the induction of CAIA by PCR using genomic DNA.

**Histopathology and C3 and IgG deposition scores in forelimbs and hind limbs**

All mice were sacrificed at day 10, after which both forelimbs and the right hind limb (knee, ankle, and paw) were processed for histopathology studies (Fig. 2A, 2B), measurement of local C3 deposition (Fig. 2C, 2D), and IgG deposition (Fig. 3). Five joints from WT, C3aR−/−, C5aR−/−, and C6-def mice used in this study were examined for inflammation, pannus formation, cartilage damage, and bone damage (Fig. 2A, 2B). Individual scores for these parameters were all significantly (p < 0.05) decreased in C3aR−/− and C5aR−/− compared with WT mice (Fig. 2A). In addition, all joint mean (AJM) scores for histopathology were significantly (p < 0.001) reduced by 44 and 57% in C3aR−/− and C5aR−/− mice, respectively, compared with the WT mice (WT, 14.07 ± 0.544; C3aR−/−, 7.86 ± 0.66; and C5aR−/−, 6.02 ± 0.17). Individual scores for inflammation, pannus formation, cartilage, and bone damage were also significantly (p < 0.05) decreased in C6-def compared with WT mice (Fig. 2B).

There was a significant decrease by 39% in the AJM histopathology scores in C6-def mice compared with the WT mice (WT, 12.07 ± 0.947; C6-def, 7.35 ± 0.73). C5aR−/− mice have significantly less inflammation versus C3aR−/− mice (p < 0.0078) and versus C6-def mice (p < 0.017), less pannus formation versus C3aR−/− mice (p < 0.012) and versus C6-def mice (p < 0.023), and less cartilage damage versus C3aR−/− mice (p < 0.041) (Fig. 2A, 2B). AJM scores were also significantly reduced in C5aR−/− mice versus C3aR−/− (p < 0.020) and versus C6-def mice (p < 0.049). No significant reduction in cartilage and bone damage was observed between C5aR−/− and C6-def mice. Representative histopathologic examples of the knee joints from WT, C3aR−/−, C5aR−/−, and C6-def mice are shown in Fig. 4A–D.

Deposition of C3 and IgG was specifically examined in the knee joints of all WT, C3aR−/−, C5aR−/−, and C6-def mice. No C3 deposition was present in the knee joints of control WT and C3−/− mice without CAIA (Supplemental Fig. 2). Similarly, no C3 deposition in the joints of all WT, C3aR−/−, C5aR−/−, and C6-def mice. At day 10 following Arthrogen injection, mice were sacrificed. Histopathologic scoring for inflammation, pannus formation, and cartilage and bone damage from five joints, including forelimbs (right and left) and one hind limb (right knee, ankle, and paw), was performed following tissue processing and Toluidine-blue staining of sections. A. Individual components of histopathologic scores from WT (n = 14), C3aR−/− (n = 13), and C5aR−/− (n = 11) mice. B. Histopathologic scores from all five joints of WT (n = 11) and C6-def (n = 8) mice. Knee joints of mice with CAIA were used to examine for C3 deposition. C. C3 deposition in knee joints in the synovium, on the surface of cartilage, and total scores (synovium plus cartilage) from WT, C3aR−/−, and C5aR−/− mice. D. C3 deposition in the synovium and on the surface of cartilage from WT and C6-def mice. All data are represented as scores (mean ± SEM). *p < 0.05 in comparison with WT mice of individual histopathologic and C3 deposition scores. **p < 0.05 intergroup comparison of individual histopathologic and C3 deposition scores between C5aR−/− and C3aR−/− or C6-def mice.

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**FIGURE 1.** CDA and prevalence of CAIA among WT, C3aR−/−, C5aR−/−, and C6-def mice. Arthrogen was injected i.p. on day 0 followed by an injection of LPS on day 3. Mice were evaluated daily by an observer blinded to the genotype of each mouse. Data are expressed as the CDA score (A, C) and prevalence of arthritis (B, D) versus days after the injection. A, CDA among WT, C3aR−/−, and C5aR−/− mice. B, The prevalence of disease in WT, C3aR−/−, and C5aR−/− mice. C, CDA in WT and C6-def mice. D, The prevalence of disease in WT and C6-def mice. Data shown in A represent the mean ± SEM based on WT, n = 14; C3aR−/−, n = 13; and C5aR−/−, n = 11. Data shown in B represent the mean ± SEM based on WT, n = 11, and C6-def, n = 8. *p < 0.05 in C3aR−/−, C5aR−/−, and C6-def mice in comparison with CDA in WT from days 5–10.

**FIGURE 2.** Histopathological scores and C3 deposition in the joints of all WT, C3aR−/−, C5aR−/−, and C6-def mice. At day 10 following Arthrogen injection, mice were sacrificed. Histopathologic scoring for inflammation, pannus formation, and cartilage and bone damage from five joints, including forelimbs (right and left) and one hind limb (right knee, ankle, and paw), was performed following tissue processing and Toluidine-blue staining of sections. A. Individual components of histopathologic scores from WT (n = 14), C3aR−/− (n = 13), and C5aR−/− (n = 11) mice. B. Histopathologic scores from all five joints of WT (n = 11) and C6-def (n = 8) mice. Knee joints of mice with CAIA were used to examine for C3 deposition. C. C3 deposition in knee joints in the synovium, on the surface of cartilage, and total scores (synovium plus cartilage) from WT, C3aR−/−, and C5aR−/− mice. D. C3 deposition in the synovium and on the surface of cartilage from WT and C6-def mice. All data are represented as scores (mean ± SEM). *p < 0.05 in comparison with WT mice of individual histopathologic and C3 deposition scores. **p < 0.05 intergroup comparison of individual histopathologic and C3 deposition scores between C5aR−/− and C3aR−/− or C6-def mice.
IgG deposition (brown color stain) on the cartilage surface is shown by black arrows. IgG deposition on the surface of cartilage from knee joints of WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice with CAIA. A. Cartilage surface in the knee joints is marked as C. IgG deposition (brown color stain) on the cartilage surface is shown by black arrows. Top panel, left to right, Cartilage surface from the knee joints of: WT mice (1) and C3aR<sup>-/-</sup> mice (2), both with CAIA. Center panel, left to right, C5aR<sup>-/-</sup> mice (3) and C6-def mice (4), both with CAIA. Bottom panel, left to right, C57BL/6 mice (5) and RAG2<sup>-/-</sup> mice (6), both without CAIA. Original magnification in all pictures was ×40 to show the surface of cartilage. B. Quantification of IgG deposition in the joints of all WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice. At day 10 following Arthrogen injection, mice were sacrificed, and IgG deposition was scored on a scale of 0–3. Endogenous IgG was considered as normal background levels and knee joints from RAG2<sup>-/-</sup> mice on a C57BL/6 background naturally lacking IgG were used as negative controls. All data represent the mean ± SEM based on n = 21 for WT, n = 13 for C3aR<sup>-/-</sup>, n = 11 for C5aR<sup>-/-</sup>, and n = 8 for C6-def mice. *p < 0.001 in comparison with WT mice by Tukey’s multiple comparison test.

Position was present in the knee joints of control C5aR<sup>-/-</sup>, C3aR<sup>-/-</sup>, and C6-def mice without CAIA (data not shown). C3 deposition in the synovium and cartilage was significantly reduced in the knee joint of C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice compared with WT (Fig. 2C) and was also reduced significantly in the synovium and cartilage of C6-def mice compared with WT mice (Fig. 2D), all with CAIA. AJM scores (synovium and cartilage) for C3 deposition were reduced by 41, 57, and 30% in C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice, respectively, compared with WT mice with CAIA. With regard to individual compartments, C3 deposition in the synovium of C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice compared with the WT mice was decreased by 37, 55, and 23%, respectively (Fig. 2C, 2D). C3 deposition on the cartilage surface of C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice compared with the WT mice was decreased by 45, 60, and 42%, respectively (Fig. 2C, 2D). The correlations (r) between AJM C3 deposition in the knee joints and CDA at the 95% confidence interval in WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def were 0.57 (p < 0.0017), 0.90 (p < 0.001), 0.65 (p < 0.027), and 0.53 (p < 0.18), respectively. There was more decrease in C3 deposition (AJM score) in the synovium as well as on the cartilage surface in the knee joint of C5aR<sup>-/-</sup> mice than in C3aR<sup>-/-</sup> (p < 0.016) and C6-def (p < 0.0027) mice (Fig. 2). Representative C3 deposition pictures of the knee joints from WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice are shown in Fig. 4E–H.

IgG deposition was specifically examined in the knee joints of all WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice. No IgG deposition was observed in the synovium of these mice. Representative IgG deposition pictures on the surface of cartilage from the knee joints of WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice are shown in Fig. 3A1–4. Representative IgG deposition pictures on the surface of cartilage from the knee joints of C57BL/6 WT mice without CAIA and RAG2<sup>-/-</sup> C57BL/6 mice without CAIA are shown in Fig. 3A5 and 6. IgG deposition on the surface of the cartilage was significantly (p < 0.001) reduced in the knee joints from C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice, but not in C6-def mice, in comparison with the WT mice with CAIA (Fig. 3B). Endogenous IgG present on the cartilage of knee joints from WT and RAG2<sup>-/-</sup> mice without CAIA was considered as the baseline background.

C3 deposition and C5a generation induced by anti-collagen Abs in vitro

Sera from WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice were used for in vitro studies on complement activation. To study activation by all three complement pathways, sera were serially diluted 2-fold from 1:10 for C3b generation, and from 1:50 for C5a generation, in buffer containing Ca<sup>2+</sup>. To analyze activation by the AP only, sera were serially diluted 2-fold in buffer in the absence of Ca<sup>2+</sup> with Mg<sup>2+</sup>/EGTA. Serial 2-fold dilutions of sera were incubated on plates with adherent anti-CII mAb, and C3 deposition on the ELISA plate and C5a generation in the supernatant were measured (Fig. 5). Negative controls included NOD sera naturally lacking C5 and sera from C3<sup>-/-</sup> mice. With Ca<sup>2+</></sup>-sufficient buffer, equivalent levels of C3 deposition were observed using all sera except for sera from C3<sup>-/-</sup> mice in which no C3 deposition was observed, as expected (Fig. 5A). Levels of C5a generation in the presence of Ca<sup>2+</sup> were slightly lower using sera from C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice in comparison with WT and absent using sera from NOD mice (Fig. 5B). In the absence of Ca<sup>2+</sup>, in which only the AP was active, levels of C3b deposition and of C5a generation were slightly lower using sera from C6-def mice in comparison with WT (Fig. 5C, 5D).

Assessment of macrophage and neutrophil infiltration in the synovium of knee joints from mice with CAIA

The infiltration of macrophages and neutrophils in the knee joint synovium from WT, C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice was determined using immunohistochemical methods with specific cell-surface markers, as outlined in the Materials and Methods. The percentages of macrophages and neutrophils were decreased significantly in the synovium of C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice with CAIA in comparison with the WT mice (Fig. 6). The decrease in synovial macrophages in C3aR<sup>-/-</sup> and C5aR<sup>-/-</sup> mice as compared with the WT mice with disease was 20 (p < 0.005) and 35% (p < 0.001), respectively. A more dramatic decrease in the percentages of synovial neutrophils was observed in C3aR<sup>-/-</sup>, C5aR<sup>-/-</sup>, and C6-def mice with disease as compared with WT mice with decreases of 56 (p < 0.014), 72 (p < 0.002), and 57%
FIGURE 4. Histopathology, C3 deposition, monocyte/macrophage infiltration, and neutrophil infiltration from WT, C3aR−/−, C5aR−/−, and C6-def mice with CAIA. Knee joints with maximum CDA scores from WT, C3aR−/−, C5aR−/−, and C6-def mice with CAIA were selected. Areas of synovium (S), cartilage (C), and meniscus (M) are identified. The black arrows all point to the synovium. From left to right, knee joints of WT, C3aR−/−, C5aR−/−, and C6-def mice stained with Toluindine-blue (blue color) are shown in A–D. Similarly, knee joints with C3 deposition (brown color) are shown in E–H; knee joints stained with F4/80 (red color) for monocytes/macrophages are shown in I–L; and knee joints stained with neutrophil (brown color) surface marker are shown in M–P. Original magnification for all pictures was ×10 (a magnification scale bar at ×10 of 10 μm [0.01 mm] is included in the lower right hand corner in all panels).

(p < 0.044), respectively. No statistically significant decrease was seen in the percentages of synovial macrophages in C6−/− mice with disease as compared with WT mice with disease (Fig. 6). Representative examples of neutrophil and macrophage infiltration are shown in Fig. 4I–L and 4M–P, respectively.

Cytokine mRNA from synovium obtained by laser capture microdissection from the knee joints of mice with disease mRNA levels were evaluated by QRT-PCR using cDNA made from the aRNA obtained using laser capture microdissection from the synovium of the left hind limb of WT, C3aR−/−, C5aR−/−, and C6-def mice (Fig. 7). No significant differences were seen in the levels of TNF-α mRNA in the synovium of C3aR−/− and C6-def mice with disease (Fig. 7A). In contrast, a significant decrease (p < 0.011) was observed in the levels of TNF-α mRNA in the synovium of C5aR−/− mice in comparison with WT mice (Fig. 7A). Significant decreases in levels of IL-1β mRNA were also seen in the synovium of C5aR−/− (p < 0.046) and C6-def (p < 0.005) mice, but not C3aR−/− mice, in comparison with WT mice (Fig. 7B).

Absolute levels of complement components in mice sera The absolute levels of various complement components such as C1q, C4, C3, factor B, and factor D were measured in sera from WT, C3aR−/−, C5aR−/−, and C6-def mice (Table I). No major decreases in the levels of complement proteins were observed that might provide alternative explanations for differences in CDA and histologic changes in these strains. However, decreases of 35, 32, and 26% in the absolute levels of C4 were seen in the sera from C5aR−/− mice in comparison with sera from WT, C3aR−/−, and C6-def mice, respectively. The decrease in the levels of C4 in sera from C5aR−/− mice compared with WT mice was significant (p < 0.014).

Discussion
The primary goal in this study was to compare the roles of the C3aR, C5aR, and MAC (C5b-9) in the pathogenesis of inflammatory arthritis using an identical mouse model, CAIA, in a single strain of C57BL/6 mice. We found that each of the three effector mechanisms initiated by the C5aR, C5aR, or the MAC was essential to the full development of arthritis; deficiency states in each of the three resulted in significant amelioration in both the primary clinical and histopathologic disease endpoints. The lack of C5aR showed the greatest decreases in CDA and parameters of histological change in these studies. Previous studies demonstrated no effect of a deficiency in the C3aR on CAIA on the BALB/c background (40) and a lack of effect of C6-def in anti-GPI–induced arthritis in the C3H/He background (7). Nevertheless, our current studies in C57BL/6 mice have demonstrated a substantial role for C3aR as well as a comparably important role of the MAC in CAIA, pointing out the importance of evaluating more than one strain of mice and model before concluding a lack of effect of a particular complement effector pathway.

We observed some key differences in the specific downstream effects of each mechanism of inflammation and tissue destruction. A significant decrease in the percentage of synovial neutrophils was observed in C3aR−/−, C5aR−/−, and C6-def mice, and a decrease in macrophages was observed in both C3aR−/− and C5aR−/−, but not in C6-def, mice. In addition, a significant decrease in TNF-α mRNA levels was observed in the synovium of C3aR−/− mice, and a decrease in IL-1β mRNA in both C5aR−/− and C6-def mice, whereas C3aR−/− mice demonstrated no changes in either cytokine. These results may reflect the effects of different expression of the two receptors or of alternate receptors for C3a and C5a, differences in signal transduction pathways that follow engagement of C3aR, C5aR, and the G-protein linked pathways that are subsequently activated, or differences in the numbers or types of infiltrating cells. However, the specific mechanisms involved in absent expression of mRNA for TNF-α and IL-1β in particular strains of gene-deficient mice remain unknown.

Neutrophil activation through C5aR in CAIA, possibly acting synergistically through enhancement in activating Fc receptors, likely plays an important role in the differential effects following chemotactic peptide engagement. This conclusion is supported by data from other experimental models of arthritis initiated by immune complexes, in which the absence of receptors for C5a on the surface of neutrophils in C5aR−/− mice likely resulted in the observed decrease in infiltration of neutrophils (39). In addition, mouse neutrophils and macrophages are known to express C5aR (41) and upon contact with human recombinant C5a mononuclear phagocytes release TNF-α and IL-1β (42). Thus, with a decrease in synovial
neutrophils and the lack of a C5aR signal, the absence of detectable mRNAs for these cytokines in our studies is not unexpected.

Alternatively, in the absence of C5aR, there may be counter-regulatory mechanisms available, such as the existence of CSL2 receptors that would interact with the available C5a. It has been shown that C5aR−/− mice that also express CSL2 receptors alone do not respond with a proinflammatory phenotype to C5a (21). This receptor may thus serve to modulate C5a biological functions in vivo. In addition to the potential effects of CSL2, blockade of C5aR on alveolar macrophages in lung Arthus reactions led to an increased ratio of FcγRIIB (inhibitory) to FcγRIII (activating) receptors (43). Therefore, local inhibition of lung C5aR can abrogate inflammation by increasing the relative expression ratio of the inhibitory receptor FcγRIIB.

Despite the major role for C5a and C5aR, deficiencies in either C3aR or MAC function in our studies also led to substantial decreases in the CDA and joint histologic injury scores. Consistent with this outcome, receptors for C3a are also present on murine monocytes/macrophages and neutrophils. It was shown that C3a stimulation of nonadherent PBMCs suppressed LPS-induced mRNA levels of TNF-α and IL-1β, whereas C3a stimulation of adherent PBMCs led to enhanced LPS-induced TNF-α and IL-1β mRNA levels (44). Thus, C3a causes cytokine release from many cell types in vitro, including IL-1β and TNF-α (44). However, the absence of changes in the levels of these cytokines in C3aR−/− mice suggests that the C5a that is generated is able to provide a sufficient signal in the absence of C3aR. Alternatively, it has also been shown that C3a binds to the receptor for advanced glycation end products (RAGE) (45). In collagen-induced arthritis, the expression of RAGE is increased, and synovial tissue inflammation, cartilage, and bone destruction are decreased by treatment with soluble RAGE (46). Therefore, the more modest decrease in disease in C3aR−/− mice might be due to the availability of alternate receptors for C3a, such as RAGE, on the surface of effector cells. In this instance, the high levels of TNF-α and IL-1β in the synovium from the knee joints of C3aR−/− with CAIA may be due in part to the binding of C3a to RAGE on macrophages.

Another unexpected result, based on prior studies of anti-GPI-induced arthritis, was our finding of protection from CAIA in the presence of C6 deficiency and the resulting absence of the MAC.
One explanation may be that, despite the influx of macrophages and the absence of TNF-α in the knee joints of C6-def mice with CAIA that was similar to WT mice, the influx of neutrophils was reduced. Thus, partial protection of C6-def mice from CAIA may be dependent on decreased neutrophils and not on macrophages. Our studies are also consistent with the results of previous studies on experimental models of arthritis in rats or myasthenia gravis in rabbits, in which it was shown that C6 deficiency effectively reduced disease severity (34, 36). An important direct or bystander role for MAC in inflammatory arthritis may be the induction of cytokines. This is supported by the minimal levels of IL-1β mRNA in the joints in C6-def mice with CAIA.

It should be emphasized that C6 deficiency was originally described in the Peru-Coppock strain, which were then backcrossed for 10 generations into the C3H/He strain (37). Thus, the C6 deficiency is due to a spontaneously occurring mutation, not to a specific induced gene deletion. The mice used in this study were obtained by backcrossing C6-def C3H/He mice into the C57BL/6 strain for eight generations. A defect in coagulation, characterized as impaired platelet aggregation, was described in C6-def C3H/He mice and was reversed in vitro by restoration with purified rat C6 protein (38). The coagulation and complement systems are known to exhibit interactions, and the possibility exists that platelet aggregation in rodents is dependent on the terminal components of the complement system. To our knowledge, a similar defect in platelet aggregation has not been examined for in C6-def C57BL/6 mice and theoretically, if present, may have influenced our observations.

One striking finding common to mice with deficiencies in mice lacking C3aR or C5aR was a decrease in local IgG and C3 deposition in the joint in comparison with WT mice. Because each effector pathway is downstream of C3 activation, it would seem that joint IgG and C3 deposition levels would be comparable between WT and mice with complement deficiencies. Nevertheless, this was not the case, suggesting that WT mice hypothetically may possess mechanisms that enhance IgG deposition with subsequent binding of C3. Thus, WT mice may exhibit increased migration of IgG into the joints and/or greater deposition of the anti-CII mAb. In other studies, C5-deficient mice failed to develop CAIA, although deposition of anti-CII mAb and C3 on the cartilage surface was unchanged (47). This finding indicates that the dependency of CAIA on C5 may be due to increased chemotaxis of neutrophils and macrophages into the joint.

The increased deposition of IgG in the joints of WT mice in the present studies, in comparison with mice with the three complement deficiencies, suggests the possibility of degradation of col-

<p>| Table I. Levels of complement components in sera from WT and complement component-deficient mice |
|-----------------------------------|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Mice (n)</th>
<th>C1q</th>
<th>C3</th>
<th>C4</th>
<th>Factor B</th>
<th>Factor D</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (23)</td>
<td>0.502 ± 0.04</td>
<td>1.730 ± 0.09</td>
<td>1.086 ± 0.09</td>
<td>0.608 ± 0.03</td>
<td>1.721 ± 0.05</td>
</tr>
<tr>
<td>C3aR−/− (15)</td>
<td>0.696 ± 0.04</td>
<td>1.674 ± 0.08</td>
<td>1.137 ± 0.08</td>
<td>0.539 ± 0.01</td>
<td>1.777 ± 0.05</td>
</tr>
<tr>
<td>p Value</td>
<td>0.002</td>
<td>0.650</td>
<td>0.668</td>
<td>0.069</td>
<td>0.449</td>
</tr>
<tr>
<td>C5aR−/− (14)</td>
<td>0.567 ± 0.03</td>
<td>1.661 ± 0.08</td>
<td>0.742 ± 0.10</td>
<td>0.500 ± 0.04</td>
<td>1.751 ± 0.14</td>
</tr>
<tr>
<td>p Value</td>
<td>0.227</td>
<td>0.571</td>
<td>0.014</td>
<td>0.0429</td>
<td>0.847</td>
</tr>
<tr>
<td>C6-def (6)</td>
<td>0.536 ± 0.06</td>
<td>1.873 ± 0.22</td>
<td>1.009 ± 0.14</td>
<td>0.567 ± 0.01</td>
<td>1.618 ± 0.06</td>
</tr>
<tr>
<td>p Value</td>
<td>0.654</td>
<td>0.563</td>
<td>0.786</td>
<td>0.249</td>
<td>0.197</td>
</tr>
</tbody>
</table>

Data are expressed as OD units with mean ± SEM based on the indicated number of sera (n) unless otherwise indicated. All p values for different complement components were compared with the corresponding values of WT mice. The p values <0.05 were considered statistically significant.

*WT mice used to measure factor B (n = 9), C3aR−/− (n = 6), and C5aR−/− (n = 6). WT mice used to measure factor D (n = 13).
lager by enzymes from phagocytic cells. This breakdown could expose new epitopes to which further anti-CII mAb could bind (47–49), leading to additional C3 fixation with further amplification of local complement activation through the AP. The explanation seems less likely that the increased IgG and C3 deposition in WT mice was secondary merely to the enhanced inflammation because these conditions would lead to further enzymatic degradation of deposited IgG and C3. A possibility also exists that IgG is processed or cleared more rapidly in C3ar−/−, C5ar−/−, and C6-def mice in comparison with WT mice. A slower rate of IgG clearance in WT mice could hypothetically lead to more deposition in joints over time. Studies are in progress to study rates of IgG clearance in these mice, as possibly suggested by the data expressed in Fig. 5.

Lastly, mice genetically deficient in a single component of the complement system may develop associated changes in other proteins. Thus, an alternative explanation for our results could be that the decreases in CDA observed in mice deficient in C3ar (Fig. 1A) or in C6 (Fig. 1C) may be secondary to decreased C5a levels in these mice, as possibly suggested by the data expressed in Fig. 5. This possibility will be explored in future studies.

In summary, we have for the first time, to our knowledge, directly compared, in a single strain of mice that are comparably backcrossed, the effects of three of the major effector pathways of complement activation that have been proposed to cause tissue injury. Notably, we determined each effector to be important, as crossed, the effects of three of the major effector pathways of complement system may develop associated changes in other proteins. Thus, an alternative explanation for our results could be that the decreases in CDA observed in mice deficient in C3ar (Fig. 1A) or in C6 (Fig. 1C) may be secondary to decreased C5a levels in these mice, as possibly suggested by the data expressed in Fig. 5. This possibility will be explored in future studies.

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Disclosures

The authors have no financial conflicts of interest.

References


