The C5a receptor has a key role in immune complex glomerulonephritis in complement factor H–deficient mice

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Chronic serum sickness leads to the formation of glomerular immune complexes; however, C57BL/6 mice do not develop glomerulonephritis unless complement factor H (CFH) is absent from the plasma. Here we studied the role for C5a receptor (R) in this setting. The exaggerated humoral immune response in CFH−/− mice was normalized in CFH−/− C5aR−/− double knockout mice, highlighting the C5aR dependence. The CFH knockout mice developed proliferative glomerulonephritis with endocapillary F4/80+ macrophage infiltration, a process reduced in the double knockout mice. There was no interstitial inflammation by histologic criteria or flow cytometry for F4/80+ Ly6ChibCCR2hi inflammatory macrophages. There were, however, more interstitial CD3+ CD4+ T lymphocytes in CFH knockout mice with chronic serum sickness, while double knockout mice had greater than 5-fold more Ly6ChibCCR2lo anti-inflammatory macrophages compared to the CFH knockout mice. Mice lacking C5aR were significantly protected from functional renal disease as assessed by blood urea nitrogen levels. Thus, IgG- and iC3b-containing immune complexes are not inflammatory in C57BL/6 mice. Yet when these mice lack CFH, sufficient C3b persists in glomeruli to generate C5a and activate C5aR.

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Pharmacologically targeting the complement system is now a clinical reality. Because of the nature of complement activation, many inhibitors directed against a given complement protein also block successive steps in the activation pathway(s). For example, the anti-C5 antibody eculizumab prevents formation of C5a and C5b-9; the latter accounts for its therapeutic efficacy and now is in routine clinical use in paroxysmal nocturnal hemoglobinuria, as well as increased risks of Neisserial infections. Eculizumab is highly effective, but it is also the only complement inhibitor in clinical use. Thus, it has been used in a number of past and present clinical trials, including in membranous nephropathy, lupus nephritis, atypical and Shiga toxin-associated hemolytic uremic syndrome, C3GN, and anti-neutrophil autoantibody-mediated GN, diseases with a considerable range in phenotype. Identifying which among the limited number of active complement products are pathogenic in a given disease process is not a trivial point. Blocking those that are not directly relevant to the disease will needlessly impair normal immunity and potential counterregulatory (anti-inflammatory) signals, for which there is growing evidence.

Here we addressed the role for C5aR in CSS-induced GN occurring in CFH–/– mice. Activation through C5aR appears to have several effects in this model, the net of which is necessary for disease.

RESULTS
C5aR-dependent acute kidney injury in CFH–/– mice with CSS
Here we used the CSS model induced with heterologous (apo)ferritin as originally described by Stilmant et al. and modified by Iskandar et al. 19 20 21 25 7 6 6 CFH–/– and CFH–/–C5aR–/– mice (n = 14 each) were actively immunized with daily intraperitoneal horse spleen apoferritin. As controls, CFH–/– (n = 4) and CFH–/–C5aR–/– (n = 8) mice were treated identically, except that apoferritin was omitted from the injections. As shown in Figure 1, control CFH–/– mice had normal renal function at the end of the 5-week experimental protocol as assessed by blood urea nitrogen (BUN) levels. In contrast, CFH–/– mice with CSS had impaired renal function (BUN = 51.4 ± 12.4 mg/dl), which was prevented in CFH–/–C5aR–/– mice (BUN = 34.0 ± 8.9 mg/dl; P < 0.001 vs. CFH–/– mice). Thus, functional renal insufficiency in the CSS model requires both the absence of CFH and the presence of C5aR.

C5aR dependence of renal inflammation in CFH–/– mice with CSS
Histopathological features of GN were evaluated at the end of the 5-week experimental protocol. As in past studies, 11 14 some control CFH–/– mice had GN scores of 0.5–1.0, as was also true of control CFH–/–C5aR–/– mice (Figure 2a). CFH–/– mice with CSS developed GN, which was significantly reduced in CFH–/–C5aR–/– mice (Figure 2a and b; P = 0.024). The primary histopathological feature was diffuse hypercellularity of the glomerular tufts (Figure 2b). The cellular composition of the observed GN was due, at least in part, to F4/80+ monocytic cells (Figure 2c). Thus, endocapillary diffuse proliferative GN in CSS appears to require absent complement regulation from CFH allowing signals through C5aR.

As typical for this model, there was no interstitial nephritis by histopathological criteria. 22 To evaluate whether there were more subtle changes in mononuclear and lymphocytic cell populations within the interstitium, a separate group of animals was studied with CSS followed by flow cytometry. F4/80+ Ly6C+ cells in tissue sites are considered macrophages, and can be divided by expression levels of Ly6C and CCR2 into inflammatory M1 (Ly6C+CCR2hi) and anti-inflammatory (or ‘alternative’) M2 (Ly6CloCCR2lo) macrophages. 23–25 Saline-treated controls had few Ly6C+CCR2hi cells in kidneys, which was also true for both groups of animals with CSS (Figure 3a and b). CFH–/–C5aR–/– mice with CSS had considerably increased intrarenal Ly6C+CCR2hi cells relative to CFH–/– control and CSS groups (Figure 3a and b). Consistent with an M2 macrophage phenotype, these cells were CD11b– F4/80+CD11c– dendritic cells (Supplementary Information online).

Peripheral blood F4/80+ cells from the same animals were also studied. Control CFH–/– mice receiving saline had distinct Ly6C+CCR2hi and Ly6C+CCR2lo populations (Figure 3b, right panel), allowing comparison with those in the kidney (Figure 3b, left panel). Interestingly, both were reduced in CFH–/– mice with CSS, along with apparent shift of the Ly6C+CCR2lo population toward higher expression of both

Figure 1 | C5aR-dependent impairment of renal function in CFH–/– mice with CSS. Blood urea nitrogen (BUN) levels were measured after 5 weeks of active immunization of CFH–/– and CFH–/–C5aR–/– mice with apoferritin or saline as controls. Data from each group were normally distributed. Shown are individual values from all mice studied, with the mean value in each group as a horizontal line. Groups were significantly different by analysis of variance (P < 0.001). *P < 0.001 vs. CFH–/–C5aR–/– plus apoferritin. CFH, complement factor H; CSS, chronic serum sickness. 
Figure 2 | Chronic serum sickness (CSS) leads to C5aR-dependent glomerulonephritis (GN) with macrophage infiltration in CFH−/− mice. Histopathological features of disease were determined after 5 weeks of active immunization of CFH−/− and CFH−/− C5aR−/− mice with apoferritin or saline as controls. (a) Semiquantitative GN scores from each mouse were compiled. Shown are individual values from all mice studied. Groups were significantly different by Kruskal–Wallis testing (P < 0.001). *P = 0.024 vs. CFH−/− C5aR−/− plus apoferritin. Representative periodic acid–Schiff staining (b) and immunofluorescence staining for F4/80+ cells (c) is shown for CFH−/− and CFH−/− C5aR−/− mice with CSS. Original magnification, ×400.

Figure 3 | Role of C5aR in interstitial macrophage (a, b) and T lymphocyte (c) numbers in CFH−/− mice after 5 weeks of active immunization with apoferritin or saline as controls. The percentage of F4/80+ kidney cells that were Ly6ChCCR2hi or Ly6CloCCR2lo is shown graphically in a with representative flow cytometry histograms shown in b. Peripheral blood mononuclear cells were studied in parallel, allowing comparisons with kidney cells. Representative data from kidney and blood are from the same animal. Similarly, the percentage of total isolated kidney cells that were CD3+CD4+ or CD3+CD8+ was determined (there was no CD3+CD4+CD8− population; c). All log-transformed data were normally distributed and used in statistical analyses. Individual values from all mice studied are shown (a, c), with y-axes as log scales and means as horizontal lines. All data are derived from the same pool of cells and are directly comparable, whereas the y-axis scales were varied based on data. All groups but Ly6ChCCR2hi cells had significantly different variances by analysis of variance (P < 0.015). Significant differences in follow-up statistical comparisons are shown individually. CFH, complement factor H.
Ly6C and CCR2. As occurred in kidneys, CFH\textsuperscript{−/−}C5aR\textsuperscript{−/−} mice had fewer Ly6C\textsuperscript{hi}CCR2\textsuperscript{hi} cells, but greater numbers of Ly6C\textsuperscript{lo}CCR2\textsuperscript{lo} cells, than CFH\textsuperscript{−/−} mice with CSS.

The percentage of intrarenal CD19\textsuperscript{+} B lymphocytes tended to be increased in CSS, with values of 9.6 (6.0-13.5), 14.8 (11.0-18.8), and 14.6 (13.1-23.5) in CFH\textsuperscript{−/−} controls, and CFH\textsuperscript{−/−} and CFH\textsuperscript{−/−}C5aR\textsuperscript{−/−} mice with CSS, respectively (NS). Relative to controls, there were increased CD3\textsuperscript{+} T lymphocytes in kidneys from CFH\textsuperscript{−/−} mice with CSS, accounted for by CD4\textsuperscript{+} cells (Figure 3c). In contrast, there was a decrease in CD8\textsuperscript{+} cells only in kidneys of CFH\textsuperscript{−/−}C5aR\textsuperscript{−/−} mice with CSS (Figure 3c). Thus, infiltration with both CD3\textsuperscript{+} CD4\textsuperscript{+} and CD3\textsuperscript{+} CD8\textsuperscript{+} T lymphocytes appears to have a C5aR dependence in the CSS model, with both being lower in kidneys of CFH\textsuperscript{−/−}C5aR\textsuperscript{−/−} mice compared with CFH\textsuperscript{−/−} mice.

**Effects of C5aR and altered humoral immune response on renal disease in CFH\textsuperscript{−/−} mice with CSS**

Glomeruli of CFH\textsuperscript{−/−} mice with CSS had immunoglobulin G (IgG) within mesangial regions with some extension to peripheral capillary walls, which was significantly reduced in CFH\textsuperscript{−/−}C5aR\textsuperscript{−/−} mice with CSS (stained red in Figure 4); respective median staining scores were 3.0 (2.0-3.0) and 2.0 (1.5-2.5; P < 0.015). As anticipated, glomeruli from CFH\textsuperscript{−/−} mice had linear glomerular capillary wall staining for C3 (stained green in Figure 4). As we have seen in prior studies,\textsuperscript{14,26,27} the baseline bright C3 staining was reduced in areas of IgG staining (Figure 4b, white arrows). Besides immunofluorescence microscopy, our past studies have shown that glomerular capillary wall complement regulation is insufficient when plasma CFH is absent; this allows ongoing complement activation in immune complex GN.\textsuperscript{20,28,29} In the experimental CSS model, C57BL/6 mice are completely resistant to glomerular inflammation unless they lack CFH, in which case they are uniformly susceptible to GN.\textsuperscript{14} This is attributable to plasma-derived CFH facilitating inactivation of C3b in glomerular-bound immune complexes, which occurs even with large immune complex burdens.\textsuperscript{30} In these instances, despite the abundance of IgG and C3b in glomeruli, which are respective ligands for inflammatory cell FcR\textsubscript{y}Rs and \(\beta_2\)-integrins, there is no glomerular inflammation. Thus, inflammatory cell recruitment to glomeruli appears to require an additional ‘signal’ on the basis of considerable circumstantial evidence, we felt this likely to be a complement activation product. Here we confirm this premise and show that C5a acting through its cognate receptor C5aR is required for this immune complex GN model.

The relevance of C5a generation and C5aR signaling in acute glomerular neutrophil infiltration has been illustrated by C5-dependent, C6-independent nephrotoxic serum nephritis in CFH\textsuperscript{−/−} mice\textsuperscript{42} and C5aR-dependent experimental anti-neutrophil autoantibody GN.\textsuperscript{30} Lipopolysaccharide is a potent inducer of C5aR expression in a variety of cells including those in the kidney,\textsuperscript{31,32} which is attributable to CCAAT and CP2 sites in the C5aR promoter.\textsuperscript{33} Matsu colleagues\textsuperscript{34} administered lipopolysaccharide along with nephrotoxic serum to generate a thrombotic microangiopathy model, which required complement activation and signals through C5aR. This model has features in common with experimental anti-phospholipid antibody syndrome, in which complement activation led to C5aR-dependent placental infiltration with neutrophils and subsequent fetal loss.\textsuperscript{35} Here, CFH\textsuperscript{−/−} mice lacking C5aR had reduced functional (i.e., BUN) and histopathological features of GN. Compared with these acute models of passive antibody administration, CSS is a relatively chronic disease process. Thus, rather than neutrophils, C5aR signals were responsible for endocapillary GN with infiltrating F4/80\textsuperscript{+} macrophages.

The CSS model is induced through repetitive immunization with apoferritin alone (i.e., without adjuvant) to generate an active humoral immune response. As in our prior studies,\textsuperscript{14} CFH\textsuperscript{−/−} mice with CSS had increased serum
levels of anti-apoferritin IgG compared with wild-type controls. We can now attribute this to excessive generation and signaling through C5aR, given equivalent anti-apoferritin IgG titers among wild-type and CFH−/−C5aR−/− mice. It has become clear that C5aR signals can affect adaptive immune responses in a variety of ways. C5aR can promote Th1 responses in T cells and inhibit effects of interferon-γ in macrophages. Thus, Wenderfer et al. showed that relative to control MRL/Faslpr lupus mice C5aR−/−MRL/Faslpr mice had diminished production of interferon-γ and interleukin-12, reduced CD4+ T-cell responses, and 15-fold less anti-double-stranded DNA IgG2a antibodies, together with lessened glomerular endo- and extracapillary inflammation and protection from renal failure.

In CSS and MRL/Faslpr models of GN, antibodies to exogenous or endogenous antigens are formed. These generate complement-activating immune complexes that deposit in glomeruli from the circulation and/or are formed directly in situ. The fact that C3 and C4 are key in immune complex processing is well established, including in these two model systems. The potential relevance for C5aR signals in glomerular immune complex processing in CSS was supported by studies by Falk and Jennette, in which C5-deficient mice had less glomerular immune complexes independent from the anti-apoferritin IgG response. There are also lower quantities of glomerular immune complexes in C5aR-deficient MRL/Faslpr lupus mice compared with C5aR-sufficient controls. Consistent with these two studies are our findings that C5aR-deficient CFH−/− mice had lower serum anti-apoferritin and glomerular-deposited IgG, which were independent from each other. An elegant paradigm advanced and supported experimentally by Gessner and colleagues is that C5aR activation of Gzα2 and phosphoinositide 3-kinase p110γ in mononuclear phagocytes leads to a transcriptional increase in FcyRIII and decrease in FcγRIIB in mononuclear phagocytes and extrinsic activation of FcγRIIB through respective gene promoter ‘C5a-inductive’ and ‘C5a-suppressive’ regions. This phenomenon is relevant in experimental disease models of liver, lung, and kidney (i.e., mesangial cells), clinically relevant is the fact that blocking C5aR signals may underlie the therapeutic efficacy of intravenous immunoglobulin. Thus, by altering the balance between activating and inhibitory FcγRs on monocytic cells, C5aR can have a considerable impact on immune complex processing and its downstream effects.

In studies by Welch et al. using the CSS model, mice given lipopolysaccharide contemporaneously developed C5aR-dependent tubulointerstitial nephritis. Although CFH is a critical glomerular capillary wall complement regulator, it appears to be dispensable in the tubulointerstitium. Thus, in our studies described here, there was little interstitial...

Figure 4 Representative immunofluorescence staining for C3 (green) and immunoglobulin G (IgG, red) in glomeruli of CFH−/− and CFH−/−C5aR−/− mice with chronic serum sickness. (a) Linear glomerular capillary wall C3 staining typical for unmanipulated CFH−/− mice was present in both groups. Mesangial IgG was present in both groups, but in greater relative amounts and with more extension to the peripheral capillary wall in CFH−/− mice compared with CFH−/−C5aR−/− mice. (b) Individual staining for C3 and IgG are shown, along with the merged image. The arrows depict regions with colocalized C3 and IgG. CFH, complement factor H.

Figure 5 Relationship between measured variables in CFH−/− (○) and CFH−/−C5aR−/− mice (○) after 5 weeks of chronic serum sickness. (a) Semiquantitative scores for glomerular immunoglobulin G (IgG; y-axis) were plotted against anti-apoferritin IgG levels (x-axis). The box enclosed by dashed lines separates the two groups completely. Measured blood urea nitrogen (BUN) concentrations (y-axis) were plotted against anti-apoferritin IgG levels (b) and semiquantitative glomerulonephritis scores (c, x-axes). The dashed lines are best-fit regression lines for data from both groups. CFH, complement factor H.
inflammation and few Ly6C<sup>hi</sup>CCR2<sup>hi</sup> (M1) macrophages in all groups. The fact that CFH<sup>−/−</sup> but not CFH<sup>−/−</sup>C5aR<sup>−/−</sup> mice with CSS have increased interstitial CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes relative to controls illustrates that this occurs in a C5aR-dependent manner, which is also true in MRL/Fas<sup>br</sup> lupus mice. The fate of interstitial CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes also appears to rely on C5aR, given that the reduction occurs only in CFH<sup>−/−</sup>C5aR<sup>−/−</sup> mice with CSS. M2 macrophages are alternative to M1 macrophages, as they tend to limit inflammation and promote repair within tissues. There were considerably more Ly6C<sup>lo</sup>CCR2<sup>lo</sup> cells in the peripheral blood and renal interstitium of CFH<sup>−/−</sup>C5aR<sup>−/−</sup> mice with CSS, indicating a blocking effect of signals through C5aR. Our ability to distinguish M1 from M2 macrophages in glomeruli is limited; as best we can determine based on F4/80 staining, glomerular inflammation is due to M1 macrophages, whereas M2 macrophages are restricted to the renal interstitium. Thus, although M2 macrophage numbers correlate with protection from clinicopathological features of GN in this model, their role(s) in this disease is not clear. Theoretically, when complement activation and C5aR signaling cease, the accumulation of M2 macrophages could be important in preserving renal function in inflammatory diseases of the different renal compartments.

Taking the current data together with our past work, glomeruli of wild-type C57BL/6 mice with CSS are normal despite the presence of abundant IgG- and iC3b-containing immune complexes. In the absence of plasma CFH, there can be active C3b to form C5 convertases. These generate sufficient quantities of C5a to provide necessary proinflammatory signals through C5aR to result in glomerular inflammation and the clinicopathological picture of GN. Downstream effects of C5aR include increasing FcγRIII quantities and allowing recognition of and activation by IgG/iC3b-bearing immune complexes in the glomerulus. Our data presented here suggest that the effects of C5aR in the active inflammatory CSS model extend beyond promoting glomerular infiltration with F4/80<sup>+</sup> macrophages. Dissecting the individual effects of C5aR signals on immune and inflammatory responses, and how each impacts renal disease, does have its experimental impediments; in particular, cells of both afferent and efferent immune systems originate from bone marrow stem cells, making chimeric studies problematic. Our ongoing research is attempting to surmount these obstacles.

### Materials and Methods

#### Animals

CFH<sup>−/−</sup> and C5aR<sup>−/−</sup> mice were generated and kindly provided by Drs. Matthew Pickering and Marina Bottro (Imperial College of London) and Drs. Allisson Humbles and Craig Gerard (Harvard Medical School), respectively. Animals were backcrossed at least 10 generations onto normal C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). Genotyping within and around CFH and C5aR alleles was performed using PCR-based approaches. All studies were approved by the University of Chicago Institutional Animal Care and Use Committee.

Two separate sets of experiments were performed with a total of 63 mice equally divided between CFH<sup>−/−</sup> (n = 31) and CFH<sup>−/−</sup>C5aR<sup>−/−</sup> (n = 32) groups. Male mice at 8 weeks of age were studied. In each experiment, littermate animals of comparable ages were studied. CSS was induced with daily intraperitoneal administration of 4 mg horse spleen apoferritin (Calzyme Laboratories, San Luis Obispo, CA). Controls (n = 8 from each group) were treated identically, except that apoferritin was omitted from the saline vehicle. After 5 weeks, animals were killed, and blood and tissues were collected. BUN concentrations were measured with a Beckman Autoanalyzer (Fullerton, CA).

#### Measurements from renal tissue

Tissues were fixed in 10% buffered formalin and embedded in paraffin, from which 4-µm-thick sections were cut and stained with periodic acid–Schiff. Slides were scored in a blinded manner by a renal pathologist (AC) for the extent of GN and interstitial nephritis using scales of 0 to 4 (in increments of 0.5) according to the schema of Passwell et al. as described previously.

Sections (4 µm) from frozen mouse kidneys were fixed in ethanol/ether (1:1) for 10 min followed by 95% ethanol for 20 min, washed with phosphate-buffered saline, and stained with fluorescein isothiocyanate anti-mouse C3 and rhodamine anti-mouse IgG (Cappel, MP Biomedicals, Solon, OH). For staining with Alexa 488 anti-mouse F4/80 (AbD Serotec, Raleigh, NC), sections were fixed twice with cold acetone (10 min, 4°C) with intervening air-drying. Slides were viewed with an Olympus BX-60 IF microscope (Carter Valley, PA). Representative photomicrographs were taken at identical settings with a Hamamatsu EM-CCD camera (Bridgewater, NJ).

Renal-infiltrating cells and peripheral blood mononuclear cells were isolated as described previously. In brief, mouse kidneys were minced and digested at 37°C for 25 min with gentle agitation with collagenase I (2 mg/ml) and DNase I (100 mg/ml) in Hank's buffered salt solution/1% (vol/vol) bovine serum albumin (all from Sigma/Aldrich, St Louis, MO). Erythrocytes were lysed with NH₄Cl, buffered salt solution/1% (vol/vol) bovine serum albumin (all from Sigma/Aldrich, St Louis, MO). Erythrocytes were lysed with NH₄Cl, and the cell suspension was passed through a 40-µm cell strainer (BD Biosciences, Franklin Lakes, NJ). Isolated cells (~10⁶) from each mouse kidney and peripheral blood were blocked with mAb 24.G2 and stained with monoclonal antibodies (with labeled fluor in parentheses) to CD3 (brilliant violet), F4/80 (AbD Serotec, Raleigh, NC), sections were fixed twice with cold acetone (10 min, 4°C) with intervening air-drying. Slides were viewed with an Olympus BX-60 IF microscope (Carter Valley, PA). Representative photomicrographs were taken at identical settings with a Hamamatsu EM-CCD camera (Bridgewater, NJ).

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#### Statistical methods

Data were analyzed using the Minitab statistical software (v. 16, College Park, MD). Data sets were first analyzed with the Anderson–Darling normality test and considered parametric with Hz > 0.05. Data from flow cytometry were log-transformed. Parametric and nonparametric data are presented as mean ± s.e.m. and median (Q1–Q3), respectively. All data presented graphically are from individual animals. Parametric data were analyzed by one-way analysis of variance with comparisons among groups using Fisher’s method. Nonparametric data sets were analyzed comparably by Kruskal–Wallis and Mann–Whitney testing. Potential correlations
among variables were first examined by calculating the Pearson product moment; those significant were further examined using the least-squares method.

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL
Figure S1. Renal M2 macrophages in CSS.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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