# IgM exacerbates glomerular disease progression in complement induced glomerulopathy

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## Abstract

While glomerular IgM deposition occurs in a variety of glomerular diseases the mechanism of deposition and its clinical significance remain controversial. Some have theorized IgM becomes passively trapped in areas of glomerulosclerosis. However, recent studies found that IgM specifically binds damaged glomeruli. Therefore, we tested whether natural IgM binds to neo-epitopes exposed after insults to the glomerulus and exacerbate disease in mice deficient in the complement regulatory protein factor H; a model of non-sclerotic and nonimmune-complex glomerular disease. Immunofluorescence microscopy demonstrated mesangial and capillary loop deposition of IgM while ultrastructural analysis found IgM deposition on endothelial cells and subendothelial areas. Factor H deficient mice lacking B cells were protected from renal damage, as evidenced by milder histologic lesions on light and electron microscopy. IgM, but not IgG, from wild-type mice bound to cultured murine mesangial cells. Furthermore, injection of purified IgM into mice lacking B cells bound within the glomeruli and induced proteinuria. A monoclonal natural IgM recognizing phospholipids also bound to glomeruli *in vivo* and induced albuminuria. Thus, our results indicate specific IgM antibodies bind to glomerular epitopes and that IgM contributes to the progression of glomerular damage in this mouse model of non-sclerotic glomerular disease.

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## INTRODUCTION

Various glomerular diseases demonstrate IgM on biopsy; however, the clinical significance of glomerular IgM remains controversial. Glomerular IgM deposition is described in diseases of widely ranging etiology such as focal segmental glomerulosclerosis (FSGS),[1] minimal change disease,[2] mesangioproliferative glomerulonephritis,[3] hypertensive nephrosclerosis,[4] and diabetic nephropathy.[5] A commonly held view is the presence of glomerular IgM represents passive trapping of the large IgM macromolecule within areas of sclerosis. Such a phenomenon would explain glomerular deposition of IgM occurring in this broad array of diseases. However, attributing glomerular IgM deposition to passive trapping does not explain the often seen co-deposition of complement protein C3, nor does it explain the absence of other proteins that are similar in molecular weight to C3 such as IgG. An alternative hypothesis is that IgM binds to specific glomerular epitopes expressed or revealed by various insults. In other words, glomerular IgM and complement activation may represent a common final pathway of injury to the glomerulus after toxic, hemodynamic, metabolic, and immunologic insults.

Several clinical observations support the hypothesis that IgM specifically binds epitopes in injured or stressed glomeruli and exacerbates disease. Studies demonstrate IgM deposition in patients with pure diffuse mesangial hypercellularity in the absence of sclerosis.[6] Furthermore, IgM is not uniformly seen in globally sclerosed glomeruli. Observational studies of patients with nephrotic syndrome and IgM deposition indicate it is a poor prognostic factor clinically. The presence of IgM is associated with steroid unresponsiveness[7, 8] and a poor response to standard immunosuppression.[9] IgM deposition in nephrotic syndrome is also associated with the development of impaired renal function[10] and is predictive of progression to end stage renal disease.[7, 9] Taken together, these observations suggest glomerular IgM is associated with more severe disease in patients with nephrotic syndrome.

Natural antibody is a class of immunoglobulin generated even without exposure to a specific target antigen. Recent research has identified several important functions of natural IgM antibody. Natural IgM binds self epitopes generated during ischemia,[11-13] aids in the removal of injured tissue,[14] and is an activator of the complement system. Thus, natural IgM appears to have both beneficial and maladaptive functions. Natural IgM helps to clear damaged and apoptotic cells and protects the individual from infection. However, natural IgM can also contribute to tissue injury through activation of the complement system and generation of tissue inflammation.

We recently demonstrated IgM contributes to glomerular disease progression in an experimental model of chemically induced FSGS.[15] In that model, the depletion of B cells reduced glomerular IgM as well as deposition of complement proteins. The decrease in glomerular IgM corresponded with an attenuated degree of albuminuria and

glomerulosclerosis. These findings support the concept that IgM binds to the damaged glomerulus, activates complement, and causes further glomerular injury. An analysis of human biopsy tissue from patients with FSGS similarly demonstrated glomerular IgM and the complement protein C3d, an activation fragment of complement. These findings indicate IgM activates complement within the glomerulus and argues against the theory that circulating IgM and C3 become passively trapped in sclerotic glomeruli.

Based on our previous work we hypothesize IgM binds neo-epitopes generated or exposed after non-scarring insults to the glomerulus and is a generalized response to glomerular injury. In order to assess IgM involvement in a non-sclerotic model of glomerular injury we utilized mice with a targeted deletion of the gene for the complement regulatory protein factor H ($fH^{-/-}$ mice). Deletion of factor H results in uncontrolled alternative complement pathway activation and glomerular injury. However, the glomerular disease is not associated with glomerulosclerosis. To test the functional importance of IgM in this model of glomerular disease we crossed $fH^{-/-}$ mice with $\mu MT$ mice, generating $fH^{-/-}/\mu MT$ double knockout mice. The $\mu MT$ mice carry a targeted disruption of the gene for the IgM μ chain.[16] Although a small population of mature B cells in these mice can produce IgG, IgA, and IgE, the mice do not produce detectable IgM.[16-18] We also injected wild-type, $\mu MT$, and $fH^{-/-}/\mu MT$ mice with IgM from wild-type mice or monoclonal natural IgM clones to determine whether IgM binds glomerular epitopes in these different strains.

## RESULTS

### IgM deposition is progressive in factor H deficient mice

Kidney sections from $fH^{-/-}$ mice at three, six, and nine months of age were assessed for glomerular deposition of IgM by immunofluorescence. Glomeruli of $fH^{-/-}$ mice demonstrated consistent IgM deposition occurring in a progressive fashion as the mice aged (representative glomeruli are shown in Figure 1a and b). Further localization of the IgM deposits demonstrated a change over time in mice with $fH^{-/-}$ mediated glomerulonephritis. In the $fH^{-/-}$ strain the areas of glomerular IgM deposition appear mesangial in distribution at three months of age (Figure 1a). At this time point the IgM deposits are distinct from glomerular C3 (Figure 1e). However, as the mice age and as disease worsens IgM deposition expands from the mesangium into glomerular capillary loops (Figure 1b and f). In contrast to IgM, glomerular IgG deposition in the $fH^{-/-}$ strain was negative. Figure 2e shows a representative tissue section from a nine month-old $fH^{-/-}$ mouse demonstrating negative staining by immunofluorescence for IgG. Wild-type mice developed minimal IgM deposition over time. Assessment of $fH^{-/-}/\mu MT$ kidney sections confirmed there was no glomerular IgM present in this strain.

### Complement components are deposited within the glomeruli of factor H deficient mice

Activated C3 fragments were present along the glomerular capillary loops of $fH^{-/-}$ mice, as previously described.[19] Glomerular C3 deposition remained constant throughout the capillary loops at both time points (Figure 1c and d). Glomerular deposition of the complement components C4 and C1q were seen in 9 month-old $fH^{-/-}$ animals (Figure 2a and c), indicating that glomerular IgM deposits activate the classical pathway in $fH^{-/-}$ mice.

Neither C4 nor C1q were detected in *fH*$^{-/-}$*/µMT* kidney sections from 9 month-old animals (Figure 2b and d).

### IgM binds to injured glomerular capillary loops

Kidney sections from nine month-old *fH*$^{-/-}$ mice were analyzed by immunofluorescence with an antibody directed against synaptopodin (a podocyte marker) and IgM, shown in Figure 3c, which demonstrates separate regions of synaptopodin and IgM staining. This is also seen on cross-section of the glomerular capillary loops, as shown in the magnified inset of Figure 3c. Further ultrastructural analysis by immunogold EM demonstrated IgM deposition on endothelial cells and in subendothelial areas in 9 month old *fH*$^{-/-}$ mice (Figure 3d).

### IgM, but not IgG, binds mesangial cells *in vitro*

A murine mesangial cell culture line (MES-13 cell line) was incubated with 10% wild-type mouse serum in PBS at 37°C and analyzed by flow cytometry to determine whether the IgM or IgG in normal mouse serum bound to the cells. As shown in Figure 4, mesangial cells demonstrated selective binding by IgM (a), but not IgG (b). We assessed a pancreatic endothelial cell line (MS-1) in similar fashion for IgM binding. These endothelial cells did not demonstrate IgM binding by flow cytometry (Figure 4c). Thus, the binding of IgM in serum appears to be cell type specific. C3 deposition was detected on the mesangial cells that bound IgM when incubated with wild-type serum (Figure 4d), demonstrating that the complement system is activated on the cell surface.

### Polyclonal IgM and specific natural IgM clones bind glomerular epitopes *in vitro*

Purified polyclonal murine IgM bound to primary culture of murine mesangial cells (Figure 5a) and to a murine mesangial cell line (MES-13 cells, Figure 5b) at 37°C. To characterize the binding of IgM to glomerular epitopes, we screened monoclonal natural IgM clones for their ability to bind to mesangial cells at 37°C. Two of the monoclonal IgM clones tested, C2 and F632, bound to mesangial cells (MES-13) *in vitro* (Figure 5b). The five other clones tested did not bind to the mesangial cells (Figure 5c). We also tested the binding of the monoclonal antibodies to mesangial cells grown in primary culture. C2 and F632 also bound to these mesangial cells, whereas the other IgM clones did not.

### Polyclonal IgM and specific natural IgM clones bind glomerular epitopes *in vivo*

To determine the glomerular binding of IgM *in vivo, fH*$^{-/-}$*/µMT* and *µMT* mice were injected intravenously with 1 mg of purified polyclonal IgM or 100 µg of monoclonal IgM clones. After 24 hours kidney sections were examined by immunofluorescence microscopy. Glomerular IgM was observed in the kidney sections from *fH*$^{-/-}$*/µMT* mice injected with polyclonal IgM (Figure 6a) but only a small amount was seen in *µMT* mice (Figure 6b). Three *µMT* mice and three *fH*$^{-/-}$*/µMT* mice were injected intravenously with 100 µg each of monoclonal IgM clone C2 or D5. Glomerular deposits of IgM along glomerular capillary walls were seen in kidney sections of all *fH*$^{-/-}$*/µMT* mice injected with C2 (Figure 6c) and D5 IgM clones (Figure 6e). Kidney sections from *µMT* mice demonstrated trace mesangial

IgM deposits following injection with the C2 IgM clone (Figure 6d) but no deposits were seen following injection with the D5 clone (Figure 6f).

Some IgM is present in the mesangium of wild-type mice. To confirm that exogenous IgM targets the glomeruli in wild-type mice, polyclonal IgM was labeled with biotin and injected into wild-type mice. After 24 hours the kidneys were harvested and the biotin label was detected in the glomeruli (Figure 7).

### Purified polyclonal and monoclonal IgM induce albuminuria *in vivo*

*μMT* mice injected with purified IgM developed an increase in albuminuria one day after injection ($P < 0.05$ by ANOVA for baseline versus day one, Figure 8a). Injection of the C2 IgM clone into $fH^{-/-}/μMT$ mice resulted in a significant increase in albuminuria at 24 hours (Figure 8b, $P < 0.05$). The C2 IgM clone did not significantly increase albuminuria in wild-type animals after 24 hours. Injection of the D5 IgM clone did not result in an increase in albuminuria in either $fH^{-/-}/μMT$ or wild-type mice (Figure 8c).

### $fH^{-/-}$ mice lacking B cells are protected from progressive glomerular disease

As seen by light microscopy the kidney sections from 9 month-old wild-type and *μMT* mice had histologically normal glomeruli with only focal areas of mild mesangial hypercellularity (Figure 9a and b, arrowheads represent areas of focal mild mesangial hypercellularity). Kidney sections from $fH^{-/-}$ animals demonstrated a large degree of hypercellularity as shown in Figure 9c. All kidney sections from $fH^{-/-}$ mice demonstrated mesangial and endocapillary proliferation. One of the $fH^{-/-}$ kidneys demonstrated crescents in two percent of the glomeruli. The $fH^{-/-}/μMT$ kidney sections had mesangial proliferation only without endocapillary proliferation (Figure 9d) and $fH^{-/-}/μMT$ kidney sections demonstrated an attenuated degree of hypercellularity compared to $fH^{-/-}$ kidneys (Figure 9e).

Double contours were also evident by light microscopy in the majority of kidney sections from $fH^{-/-}$ mice (Figure 9c, arrows identify double contours, also seen in magnified inset). There were no double contours seen in the wild type or *μMT* kidneys (Figure 9a and b). The presence of double contours was significantly lessened in the glomeruli from $fH^{-/-}/μMT$ animals compared to $fH^{-/-}$ mice (Figure 9d and f). None of the kidney sections from wild type, *μMT*, $fH^{-/-}$, or $fH^{-/-}/μMT$ demonstrated any global sclerosis.

At 9 months, $fH^{-/-}/μMT$ double knockout mice demonstrated reduced ultra-structural pathology by electron microscopy compared to the $fH^{-/-}$ mice. The $fH^{-/-}$ mice demonstrated numerous sub-endothelial and intramembranous electron-dense deposits (Figure 10a, areas of electron dense deposits marked with arrowheads), lesions of basement membrane duplications (identified by double-headed arrows), and mesangial interposition (marked with arrow), and hypercellularity (asterisk marks monocytes and/or endothelial cells). In contrast, kidney sections from $fH^{-/-}/μMT$ mice had relatively small sub-endothelial deposits (Figure 10b, deposits marked by arrowheads), otherwise the glomeruli had normal appearance by electron microscopy. Kidney sections from wild-type animals demonstrated normal glomerular ultra-structure.

At 9 months, evaluation of $fH^{-/-}/\mu MT$ mice for clinical markers of renal disease demonstrated attenuation of albuminuria compared to age-matched $fH^{-/-}$ mice, although this did not achieve statistical significance (Figure 11). Renal function appeared normal in all of the strains as assessed by serum markers (serum urea nitrogen and creatinine). Wild-type, $\mu MT$, $fH^{-/-}$, and $fH^{-/-}/\mu MT$ mice had no significant difference in blood urea nitrogen or creatinine values.

## DISCUSSION

Despite the presence of IgM in a variety of distinct glomerular diseases, the functional consequences remain a source of debate. In the current study the involvement of IgM in a non-sclerotic and non-immune complex model of glomerular disease was assessed using mice with a targeted genetic deletion of factor H. Both *in vivo* and *in vitro* we demonstrated IgM bound glomerular epitopes, but IgG did not. Based on our analysis at early compared to late stages of disease we demonstrated that complement-mediated injury of the glomerular capillaries was associated with deposition of IgM in the glomerular capillary walls as the mice aged. Deposition of IgM in the capillary walls of $fH^{-/-}$ mice as they age could be caused by the generation of neoepitopes at this location or by an impaired ability of injured glomerular cells to remove and metabolize deposited immunoglobulin and complement proteins. Mice unable to produce IgM ($fH^{-/-}/\mu MT$ mice) developed milder disease than $fH^{-/-}$ mice in terms of histologic lesions. Furthermore, the injection of B cell deficient mice with exogenous IgM induced transient albuminuria confirming a functional role for the glomerular IgM.

We also tested a panel of IgM monoclonal antibodies and found two clones, C2 and F632, which bound to mesangial cells *in vitro*. The pattern of binding was consistent across commercially available and primary mesangial cell lines. Injection of the C2 and D5 IgM clones demonstrated these clones deposit in glomerular capillary loops of mice with glomerulonephritis ($fH^{-/-}/\mu MT$ mice) but do not bind within the capillaries of wild-type mice. Furthermore, the C2 IgM clone, but not the D5 clone, induced albuminuria in the $fH^{-/-}/\mu MT$ mice, indicating the glomerular binding patterns and the pathogenicity of IgM antibodies appear to be different among distinct IgM clones.

In addition to supporting the concept that glomerular IgM binding is a specific process, these results may be useful for identifying the glomerular epitopes to which IgM binds. The C2 IgM clone was previously shown to bind to certain phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and cardiolipin, but not to proteins.[20, 21] Natural IgM has also been shown to bind to phospholipids on apoptotic T cells.[22] It is important to note that natural IgM is polyreactive, so it is possible the C2 IgM clone binds to a different epitope in this particular disease model.

Our data suggest progressive glomerular IgM deposition plays an active role in exacerbation of proteinuric glomerular disease. Glomerular damage may expose or generate neo-epitopes. IgM specific for these epitopes then binds within the damaged glomerulus and further contributes to disease progression and albuminuria. IgM binding within the glomerulus in conjunction with the local activation of complement could play a key role in the disease

progression from a variety of primary glomerular insults. The detection of glomerular IgM and C3 on kidney biopsies may, therefore, identify an important subset of patients in whom the innate immune system contributes to disease progression. Furthermore, IgM and the complement system could represent novel therapeutic targets for slowing glomerular disease progression.

A limitation to our study is the mild nature of renal injury in the $fH^{-/-}$ mice. Although histologic injury and albuminuria were attenuated in B cell deficient mice, not all of the endpoints achieved significance. We believe the binding of IgM within the glomerulus is a secondary phenomenon and cannot account for all of the renal damage. Thus, in a mild model of glomerular injury the degree of protection will be modest. Interestingly, a previous study demonstrated that the glomerular C3 deposits converted to a mesangial pattern when $fH^{-/-}$ mice were reconstituted with purified factor H.[23] The mesangial C3 deposition that emerged after factor H was restored may have been be due to the presence of mesangial IgM deposits. Based upon our results it is also possible that the efficacy of purified factor H for reversing glomerular disease in this model will be lost once sufficient IgM is deposited in the glomerulus, a concept that we will test in future experiments. Previous studies have shown there is sufficient complement activation in these mice to mediate antibody-mediated glomerular injury.[24] A receptor for IgM was also recently described[25] and it is possible that some of the pathologic effects of glomerular IgM are mediated through the interaction with this receptor and not through complement activation. However, our data demonstrated specific IgM clones bound glomerular cells while other clones did not bind. This suggests these clones are directed to a specific target epitope. If binding were occurring via the Fc receptor for IgM, we would anticipate that all of the monoclonal IgM antibodies would bind. Nevertheless, this model allows us to identify a secondary pathogenic role of IgM in a disease that is generally regarded as not involving immunoglobulin.

Debate remains as to the clinical significance of IgM deposition in glomerular disease. Clinical studies of patients with nephrotic syndrome demonstrate conflicting evidence for the contribution of glomerular IgM to the disease process. Some demonstrated more severe manifestations of nephrotic syndrome in association with glomerular IgM deposition (steroid unresponsiveness, impaired renal function, and progressive disease).[7-10] However, not all have demonstrated a significant role for IgM in nephrotic syndrome.[26, 27] It is also clear not all patients with glomerular disease develop IgM and complement deposition. However, it may be deposition of IgM and complement identifies a subset of patients in whom these molecular mechanisms are engaged. The current study shows binding of IgM within the glomerulus can be a downstream event occurring secondary to glomerular damage. This may serve as part of the repair process to help remove apoptotic cells but may also result in local tissue damage. Even though the involvement of IgM may occur in a subset of patients with any given glomerular lesion, in aggregate IgM specific for glomerular epitopes may be a common cause of progressive injury across a large number of different glomerular diseases.

Most of the immunosuppressive agents commonly used to treat patients with glomerular disease (such as corticosteroids, cyclophosphamide, and cyclosporine) do not inhibit innate immune factors such as natural antibody generation and the complement system.[28-30]

Furthermore, even though the B-1 cells that generate natural IgM express CD20 they are resistant to depletion with rituximab.[31] Consequently, standard immunosuppressive agents may not be effective at blocking these innate immune mediators' role in disease progression. This may explain the resistance to standard immunosuppressive agents seen in groups of patients with glomerular disease. It may also explain the variability in the degree of steroid responsiveness reported in clinical studies of nephrotic patients with IgM deposition. A better understanding of these disease mechanisms is needed to develop more effective therapeutics. Agents that block these innate immune factors could have the potential to slow glomerular disease progression without unnecessarily impairing adaptive immune functions.

In conclusion, this study indicates a functional role for IgM in the progression of this murine model of non-sclerotic glomerular disease. The applicability of the findings in this study needs to be confirmed in humans. Although, the presence of glomerular IgM and complement proteins in patients with a variety of glomerular diseases suggests a similar process may occur. Glomerular injury may lead to expression of *de novo* antigens or exposure of hidden antigens for which there is an innate immune response. If so, IgM may be an amplifying mechanism in many forms of glomerular disease after a wide range of triggering insults, including immunologic and non-immunologic injury of the kidney.

## MATERIALS AND METHODS

### Derivation of *fH*$^{-/-}$, *μMT*, and *fH*$^{-/-}$/*μMT* mice

Experimental cohorts included male wild type, factor H knockout (*fH*$^{-/-}$), and *fH*$^{-/-}$/*μMT* double knockout mice on a C57BL/6 background. Factor H knockout mice were provided by Matthew Pickering and generated as previously described.[19] The *μMT mice (*Jackson ImmunoResearch, West Grove, PA, strain 002288) and the *fH*$^{-/-}$ mice were each backcrossed onto a C57BL/6 background for nine generations. The mice were then inter-crossed to generate the double knockout *fH*$^{-/-}$/*μMT*$^{-/-}$ mice. Animals were harvested at three to nine months of age. All mice were maintained at the University of Colorado Center for Laboratory Animal Care in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

### Albumin, serum urea nitrogen, and creatinine measurements

Urine albumin was determined by ELISA according to manufacturer's instructions (Bethyl Laboratories, Inc, Montgomery, TX). Serum urea nitrogen and serum and urine creatinine were determined by automated analysis with the AlfaWassermann ACE® Clinical Chemistry Analyzer.

### Renal histology

Kidney tissue was fixed in formalin, embedded in paraffin, cut into 4 μm sections, and stained with periodic acid-Schiff and Masson's trichrome stain for evaluation by light microscopy. Sections were assessed for the extent of hypercellularity, double contours, and glomerulosclerosis. Histologic analysis was performed by a renal pathologist blinded to study groups. Fifty glomeruli per animal per section were analyzed. The average

involvement of glomerular lesions of the 50 glomeruli examined per animal is represented as a single data point in Figure 9 panels e and f.

Additional kidney tissue for electron microscopy was fixed in 2% glutaraldehyde / 2% paraformaldehyde with 0.1 M cacodylic acid and 3% sucrose, embedded in resin, and cut into ultrathin sections. Three animals per group and eight to twelve glomeruli per animal were analyzed by a renal pathologist blinded to study groups.

### Immunofluorescence microscopy

Snap-frozen kidney sections (5 μm) were stained with Cy3-conjugated goat antibody against mouse IgM (Jackson ImmunoResearch) or FITC-conjugated goat antibody against mouse C3 (MP Biomedicals) and assessed by immunofluorescence as previously described.[15] Staining with a rabbit antibody against mouse synaptopodin (Sigma, St. Louis, MO) was used to localize podocytes followed with a secondary FITC-conjugated donkey antibody against rabbit IgG (Jackson ImmunoResearch). Appropriate secondary antibodies and isotype controls were obtained from Jackson ImmunoResearch.

High-powered images of 25 glomeruli per animal per section were obtained on an Olympus BX51 microscope. The relative fluorescence units were measured by ImageJ software and image overlays were created in Adobe Photoshop CS3 (San Jose, CA). The measured relative fluorescent units for each glomerulus were normalized by correcting for the background fluorescence on the image. The average value of the normalized relative fluorescent units from the 25 glomeruli per animal was used for assessment.

### Immunogold electron microscopy

Snap-frozen kidney sections (10 μm) were fixed in 10% formaldehyde for 30 minutes and permeabilized in 0.1% Tween 20 in TBS (0.05 M Tris, 0.9% NaCl, pH 8.2) for 1 hour. The sections were incubated in colloidal gold conjugated goat anti-mouse IgM (British Biocell International, Madison, WI) at 1:20 dilution at 4°C overnight. After washing in TBS for 30 min, the sections were fixed in 1% glutaraldehyde for 10 min, dehydrated in a graded series of ethanol, embedded in Epon, thin-sectioned, and stained with 7.7% uranyl acetate in water and lead citrate solution (2.66% lead nitrate, 3.52% sodium citrate in CO2-free double - distilled water, pH 12) for transmission electron microscopy.

### Murine glomerular mesangial cell culture and flow cytometry

Immortalized murine glomerular mesangial cells (MES-13 cells, from ATCC, Manassas, VA), primary culture of mesangial cells from C57BL/6 mice, and immortalized murine pancreatic endothelial cells (MS-1, from ATCC) were used for flow cytometric analysis.

Primary cultures of mesangial cells were obtained from wild-type two month old C57BL/6 mice as previously described.[32, 33] Kidneys were removed under aseptic technique and cortical tissue was forced through 200 grade mesh. Unencapsulated glomeruli were collected by centrifugation and digested with type I collagenase (Sigma-Aldrich) at 37°C. The glomerular suspension was plated in RPMI 1640 media with 17% FBS, 0.1 Unit/mL insulin (Gibco), and Penicillin-Streptomycin (Gibco) and allowed to grow at 37°C in 5% $CO_2$ in air.

Characterization of primary mesangial cells demonstrated typical stellate morphology, positive smooth muscle actin staining (Santa Cruz), but were negative for synaptopodin (Sigma) and negative for CD31 (an endothelial cell marker, BioLegend).

Mesangial cells were incubated with 10% wild-type mouse serum in PBS, purified IgM, or vehicle control (PBS) for 30 minutes at 37° C. Cells were washed with PBS and then stained with PE-conjugated rat antibody against mouse IgM, isotype control (PE-conjugated rat IgG isotype control) (Novus, Littleton, CO) or Cy5-conjugated donkey antibody to mouse IgG (Jackson ImmunoResearch). Flow cytometric analysis was performed on a FACS Calibur machine (BD Biosciences, San Jose, CA) and analyzed by CellQuest Pro software (BD Biosciences).

### IgM purification and intravenous injection

IgM antibody was purified from wild-type mouse serum. Large-scale purification was performed using a HiTrap Protein G column to remove IgG (Amersham Biosciences, Piscataway, NJ). Proteins were precipitated with polyethylene glycol (Sigma) and the product was run over a size exclusion column to isolate the IgM fractions. The purity of the isolated IgM was established by Coomassie staining and Western blot analysis. The IgM was biotinylated using a EZ-link Sulfo-NHS Biotinylation Kit (ThermoScientific) according to the manufacturer's instructions, and binding of the biotinylated IgM to mesangial cells was confirmed by flow cytometry. Monoclonal natural IgM hybridomas were generated from the B cells of C57BL/6 mice as previously described.[34] Mice were injected with 1 mg of purified polyclonal IgM or 100 μg of the IgM monoclonal antibodies intravenously by tail vein (N=3 for each antibody preparation). For mice injected with polyclonal IgM, urine and serum samples were collected at baseline and on days 1, 2, and 3 following injection. Kidney tissue was obtained on days 1 and 3. Mice injected with the IgM monoclonal antibodies were sacrificed after 24 hours and the kidney tissue was analyzed.

### Statistical Analysis

Statistical analysis between treatment groups was assessed by unpaired t test or by ANOVA for multiple groups using GraphPad Prism software (La Jolla, CA). A P value less than 0.05 was considered significant.

## ACKNOWLEDGMENTS

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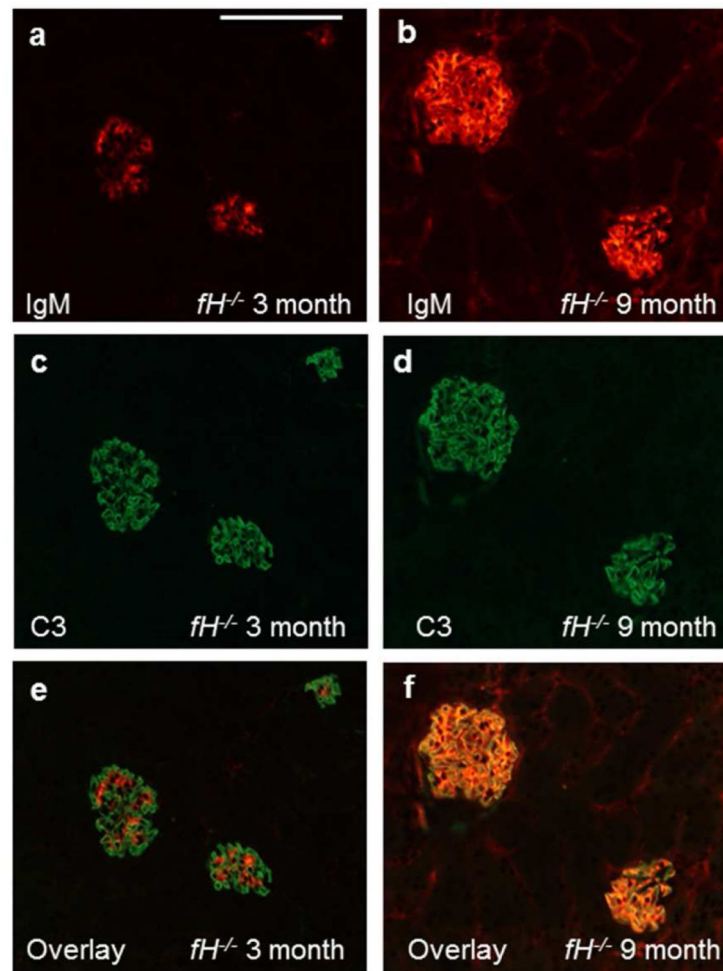
**Figure 1. Factor H deficient mice demonstrate progressive IgM deposition within the glomerulus over time**

Glomerular IgM and C3 deposition were assessed by immunofluorescence microscopy of kidney sections from $fH^{-/-}$ mice. (**a** and **b**) IgM is seen in the mesangium of young mice (3 months old). IgM deposition expands into capillary loops in older mice (9 months old). (**c** and **d**) Glomerular deposition of C3 appears in glomerular capillary loops at both ages. (**e** and **f**) Dual staining with IgM and C3 demonstrate distinct locations of IgM and C3 in $fH^{-/-}$ mice at 3 months of age and diffuse deposition of IgM at 9 months of age. Representative glomeruli from mice in each group are shown. Original magnification × 200. Bar = 100 μm.
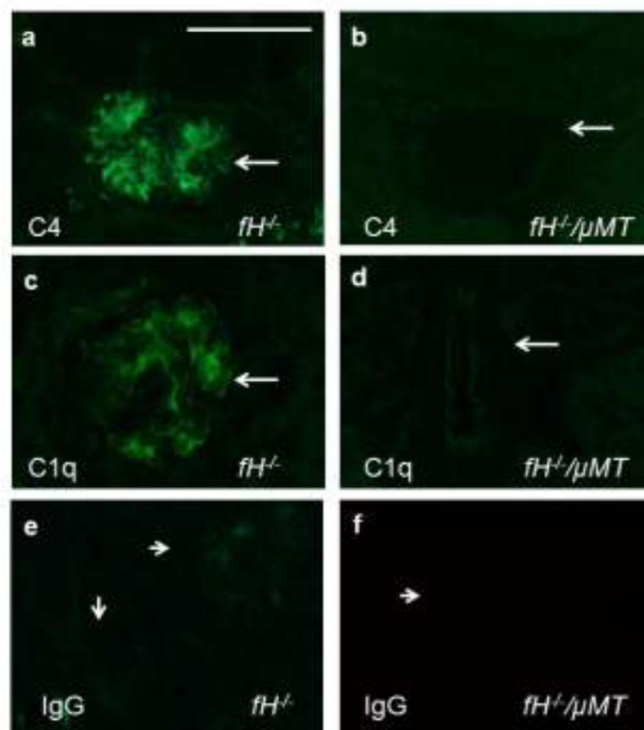
**Figure 2. Complement components are deposited within glomeruli of factor H deficient mice**
Glomerular deposition of C4 (**a**) and C1q (**c**) are seen in *fH*$^{-/-}$ mice. However, *fH*$^{-/-}$*/μMT*
mice did not demonstrate glomerular C4 (**b**) or C1q (**d**) deposition. Neither *fH*$^{-/-}$ or
*fH*$^{-/-}$*/μMT* mice demonstrated glomerular IgG deposition (**e** and **f**). Arrows show location
of glomeruli. Original magnification × 400 and bar = 100 μm.
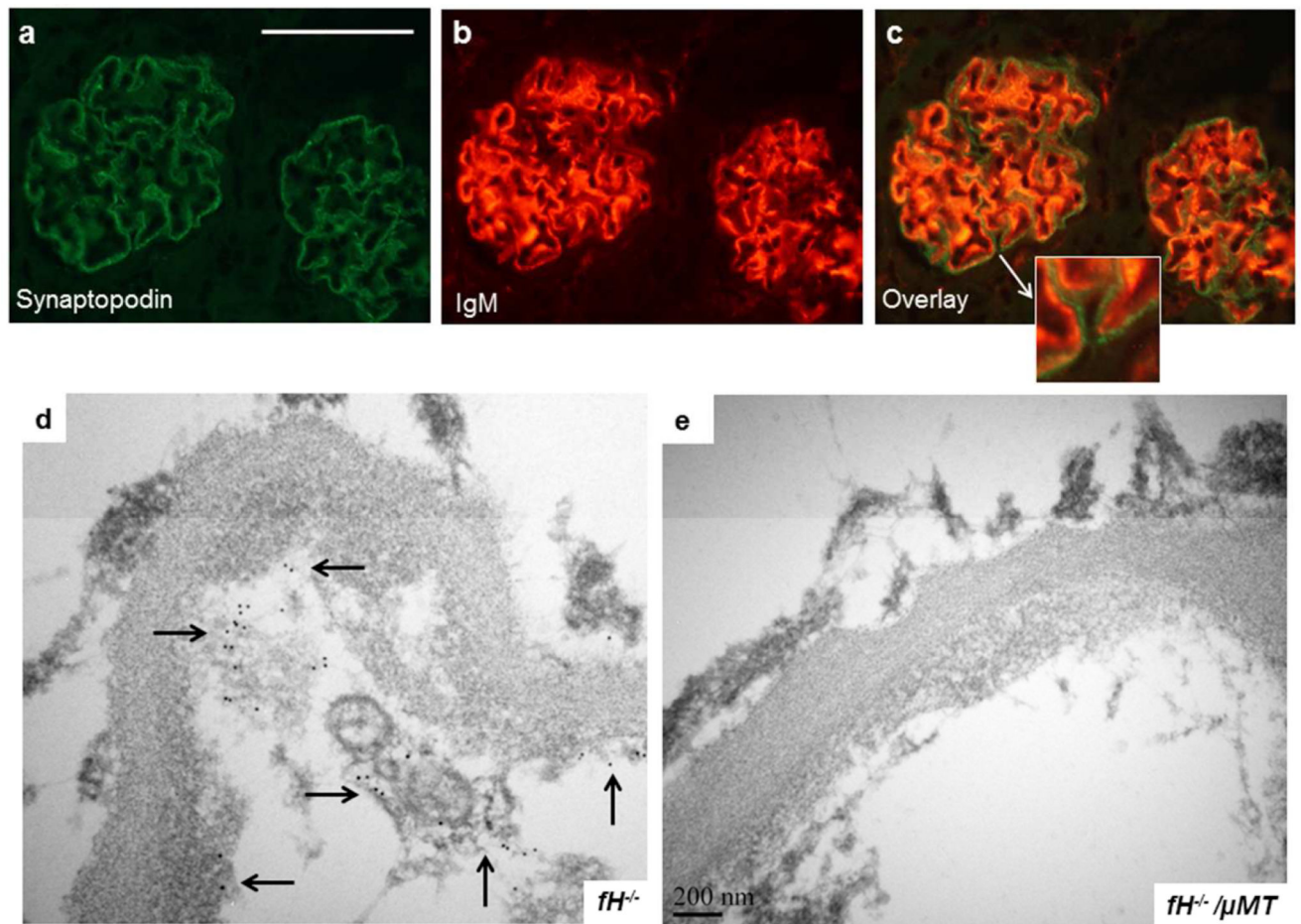
**Figure 3. IgM deposits within glomerular capillary loops of diseased glomeruli**

Kidney sections from 9 month-old *fH*$^{-/-}$ mice were assessed by immunofluorescence for distribution of IgM within the glomerular capillary loops. (**a**) Synaptopodin, a marker for podocytes is shown. (**b**) Glomerular IgM staining is shown. (**c**) Dual staining of synaptopodin and IgM demonstrates distribution within separate compartments of the capillary loops. (**d**) Immunogold electron microscopy staining for IgM demonstrates IgM deposition occurring on endothelial cells and in the subendothelial area (arrows show locations of gold beads and IgM). (**e**) Kidney sections from *fH*$^{-/-}$/*μMT* animals lacked IgM deposition. Representative glomeruli are shown. Original magnification × 400 and bar = 100 μm for panels a, b, and c. Bar = 200 nm for panels d and e.
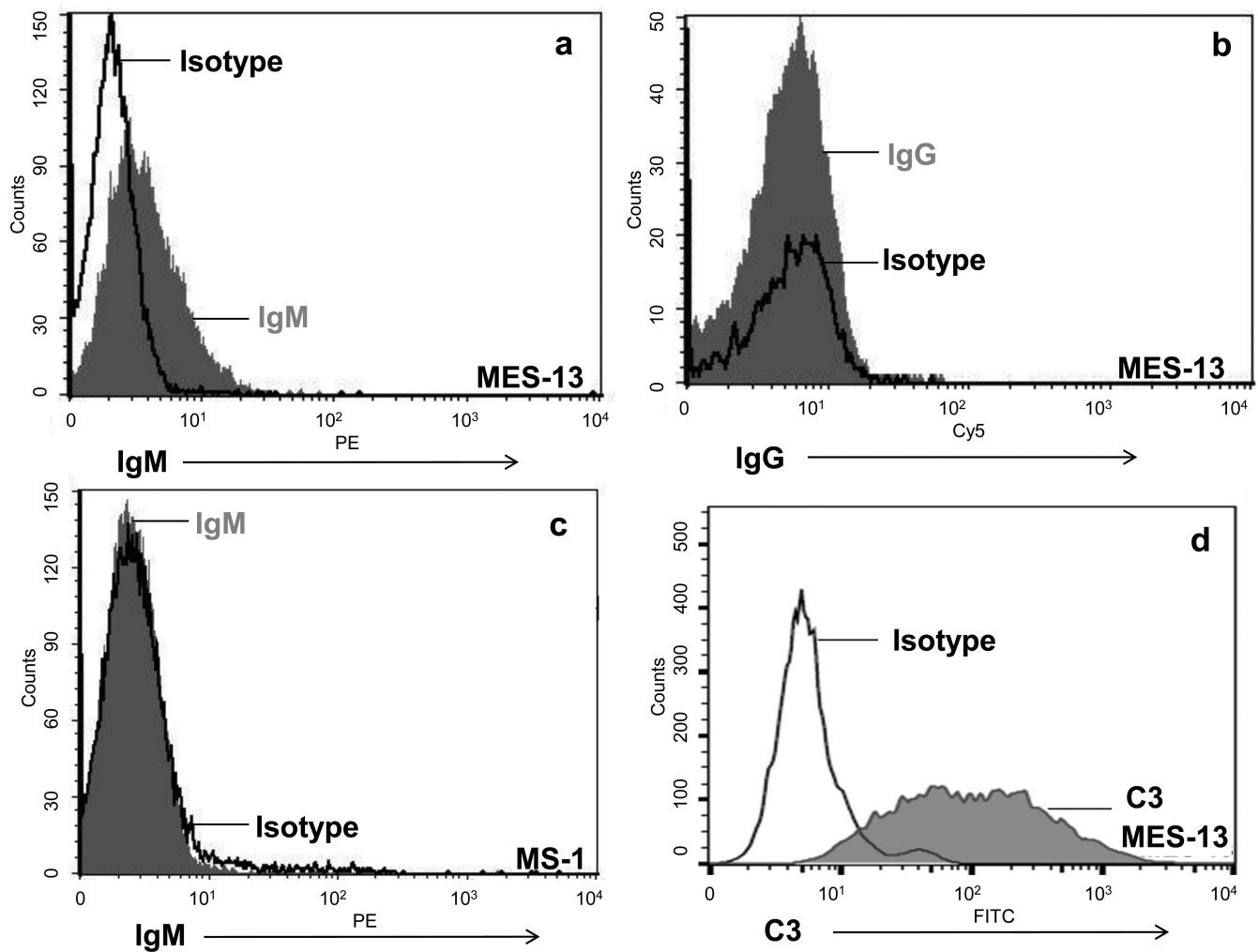
**Figure 4. IgM binds to mesangial cells *in vitro***
Cultured murine mesangial cells were incubated with normal mouse serum at 37°C. Cells were analyzed by flow cytometry for the presence of IgM, IgG, and C3. (**a**) IgM binds to mesangial cells (MES-13) (positive staining demonstrated by grey shaded histogram and isotype control represented by black outline). (**b**) IgG does not bind to mesangial cells. (**c**) IgM binding was specific to mesangial cells, as a pancreatic endothelial cell line (MS-1) did not bind IgM. (**d**) Mesangial cells that bound IgM also bound C3 when incubated with mouse serum.
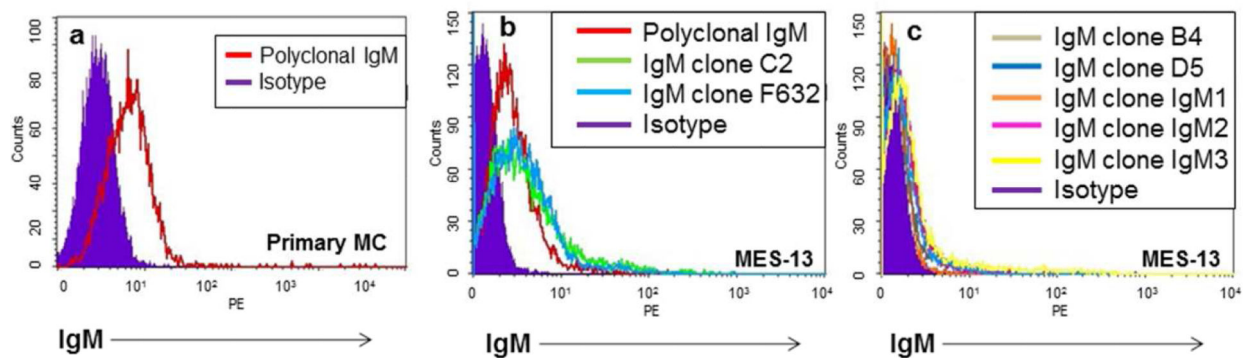
**Figure 5. Purified polyclonal and monoclonal IgM binds glomerular cells *in vitro***
Cultured murine mesangial cells were incubated with polyclonal IgM or seven different monoclonal IgM clones at 37°C. Following incubation cells were analyzed by flow cytometry to determine the degree of IgM binding. (**a**) Purified polyclonal IgM bound to cultured primary mesangial cells (Primary MC) and bound to a mesangial cell line (MES-13, panel b). (**b**) Two of the monoclonal IgM clones exhibited positive binding to mesangial cells (the monoclonal IgM clone C2 in green and the monoclonal IgM clone F632 in blue). (**c**) The remaining five monoclonal IgM clones did not demonstrate binding to mesangial cells. Isotype control is represented by the purple shaded histogram.
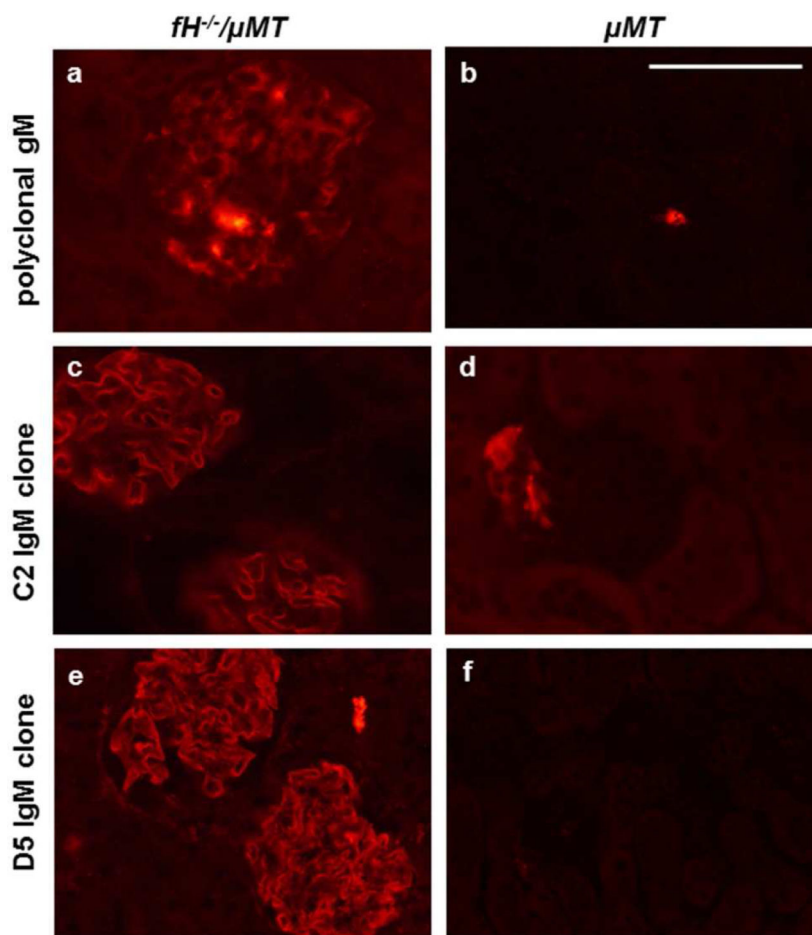
**Figure 6. Purified polyclonal and monoclonal IgM binds glomerular cells *in vivo***
*fH*$^{-/-}$*/μMT* and *μMT* mice injected intravenously with either purified polyclonal IgM or monoclonal IgM clones. Kidney sections were assessed for the presence of IgM by immunofluorescence microscopy. Glomeruli from *fH*$^{-/-}$*/μMT* animals demonstrated deposition of polyclonal IgM (**a**), the C2 IgM clone (**c**), and the D5 IgM clone (**e**). The kidney sections from *μMT* animals demonstrated trace IgM staining following injection of the C2 IgM clone (**d**) and lacked IgM deposition following injection of polyclonal IgM and the D5 IgM clone (**b** and **f**). Representative glomeruli are shown. Original magnification × 400. Bar = 100 μm.
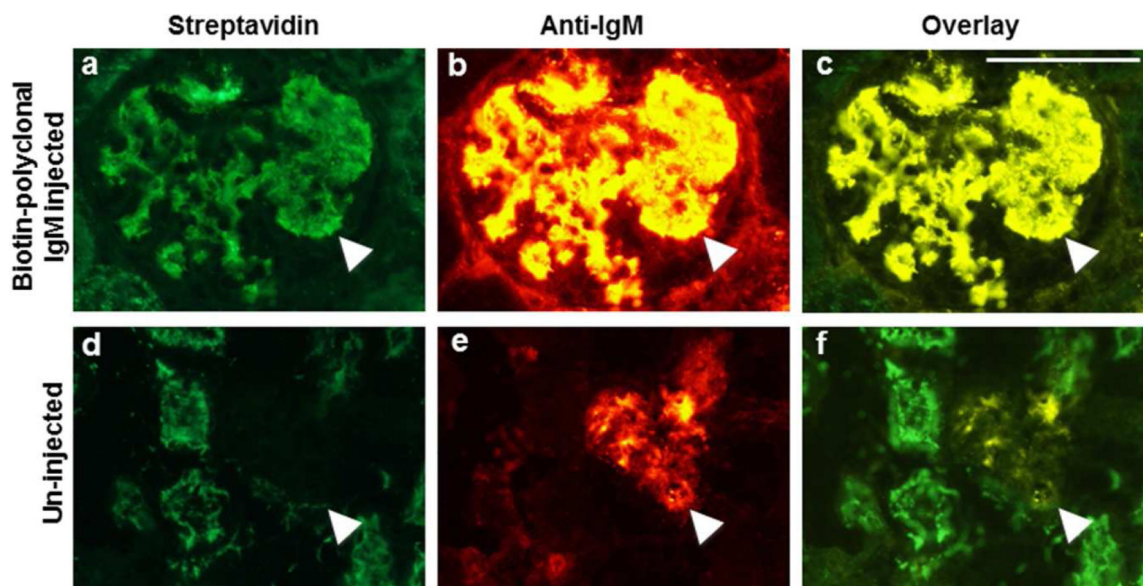
**Figure 7. Purified polyclonal IgM binds the glomeruli of wild type mice**

Seven month-old wild type (WT) mice were intravenously injected with purified polyclonal IgM conjugated with biotin (Figure 7, Biotin-polyclonal IgM injected). The kidneys were harvested at 24 hours and were assessed for polyclonal IgM deposition by immunofluorescence microscopy. Evidence of extensive polyclonal IgM deposition (detected using streptavidin-Alexa488) was observed in the glomeruli of injected mice (**a**), but not in those of un-injected mice (**d**). Staining specific for the IgM μ chain indicated the presence of mesangial IgM (both purified polyclonal and endogenous forms) within the glomeruli of injected (**b**) and un-injected (**e**) mice. Confirmation of significant polyclonal IgM deposition in the glomeruli of injected (**c**), but not in the un-injected (**f**) mice is shown in overlaid images. Representative glomeruli from each group are shown and indicated by arrowheads. Original magnification × 400. Bar = 100 μm.
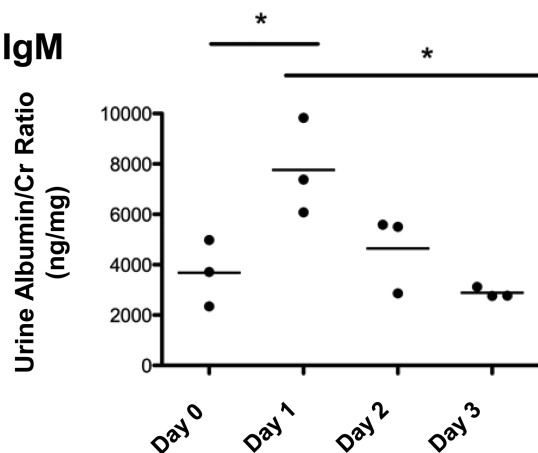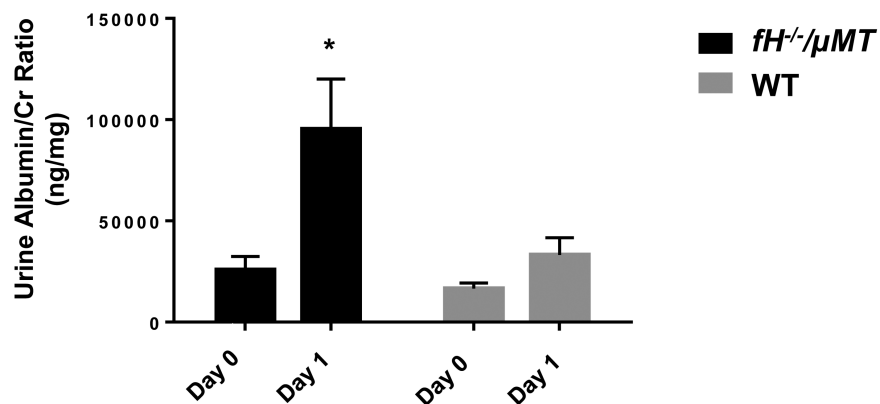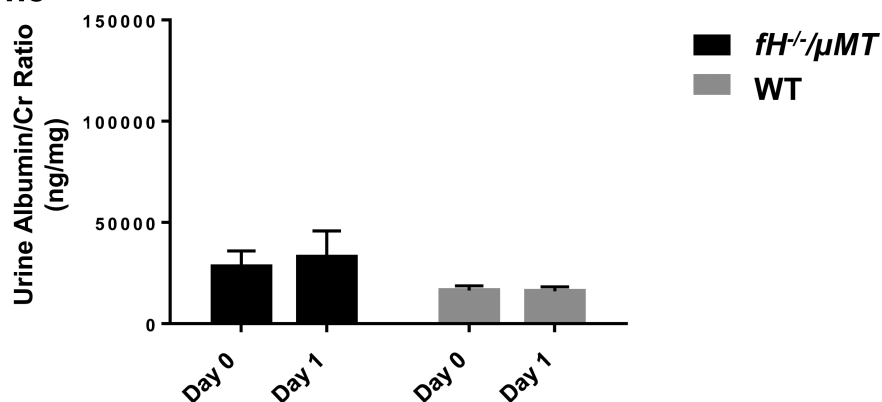
**Figure 8. Purified polyclonal and monoclonal IgM induce albuminuria *in vivo***

(**a**) Intravenous injection of purified IgM into B cell deficient (*µMT* mice) resulted in a significant increase in albuminuria at one day after injection (* $P < 0.05$ by ANOVA for Day 0 versus Day 1 and for Day 1 versus Day 3). Individual data points are presented. Lines represent means. (**b**) Injection of the C2 IgM clone significantly increased the degree of albuminuria in *fH*$^{-/-}$/*µMT* animals at Day 1 but did not induce albuminuria in wild type (WT) animals. * $P < 0.05$. (**c**) Injection of the D5 IgM clone failed to induce albuminuria in either *fH*$^{-/-}$/*µMT* or WT animals.
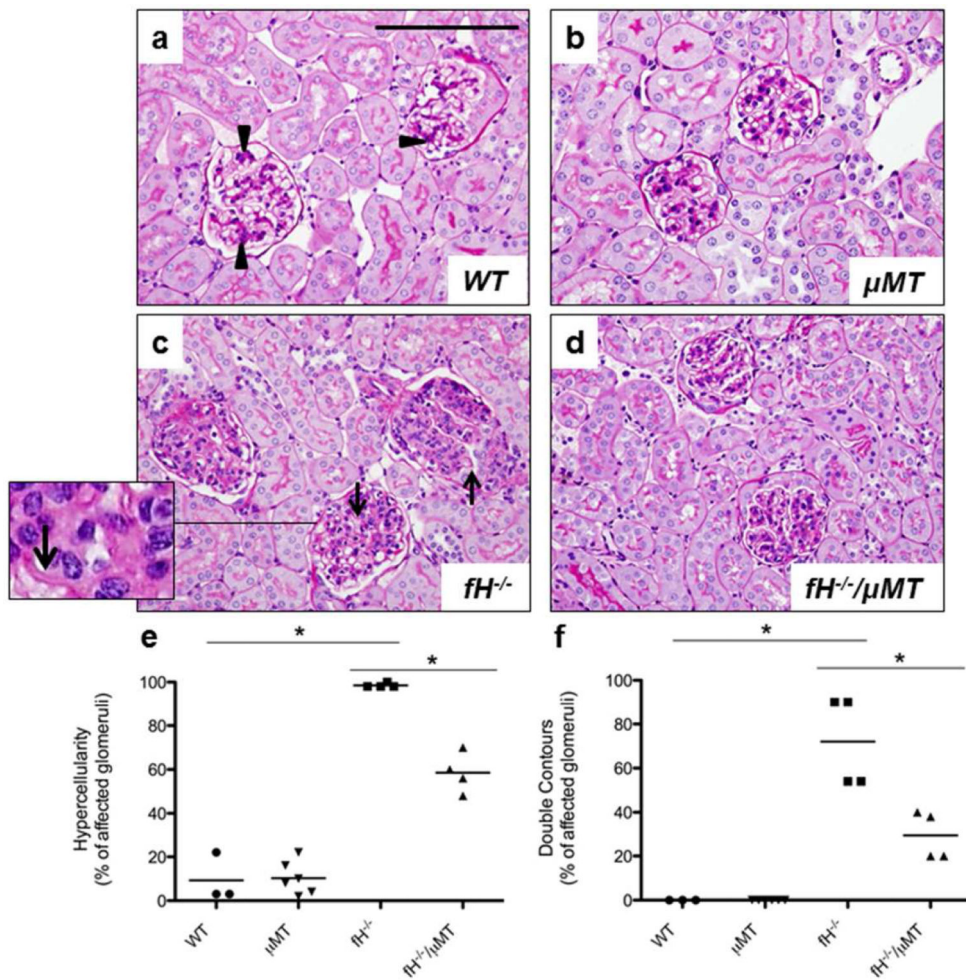
**Figure 9. Factor H deficient animals lacking B cells develop fewer pathologic lesions than factor H deficient animals by light microscopy**

(**a** and **b**) PAS stained kidney sections from 9 month-old wild type (WT) and a *μMT* mouse show two normal appearing glomeruli with only mild focal mesangial hypercellularity (arrowheads). (**c**) The kidney section from a *fH⁻/⁻* mouse demonstrates three glomeruli with global endocapillary proliferation, mesangial hypercellularity, and double contours (double contours marked with arrows, also shown in magnified inset). (**d**) In *fH⁻/⁻/μMT* mice these pathologic lesions were attenuated. Two glomeruli from a *fH⁻/⁻/μMT* kidney section are present with moderate mesangial hypercellularity and no double contours. Representative glomeruli from mice in each group are shown, original magnification × 400. Bar = 100 μm. (**e** and **f**) Quantitative assessment demonstrated a significantly higher degree of hypercellularity and double contours in kidney sections from *fH⁻/⁻* mice compared to wild type or *μMT* mice. The kidney sections from *fH⁻/⁻/μMT* mice had significantly attenuated pathologic lesions (both hypercellularity and double contours) compared to *fH⁻/⁻* mice. * $P < 0.05$ by ANOVA. Individual data points are presented. Lines represent means.
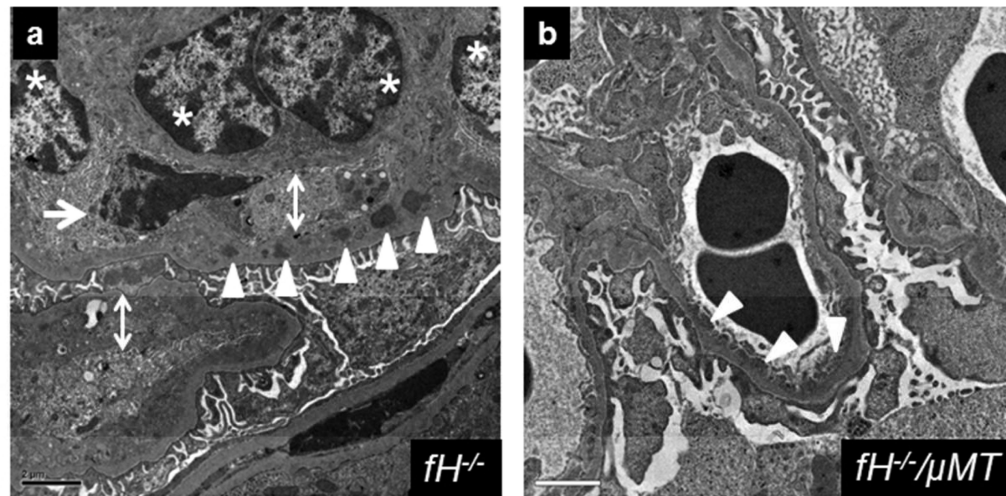
**Figure 10. Factor H deficient animals lacking B cells demonstrate milder pathologic lesions than factor H deficient animals by electron microscopy**

(**a**) Electron microscopy of kidney sections from 9 month-old *fH*−/− mice demonstrate numerous sub-endothelial and intramembranous deposits (arrowheads), mesangial interposition (arrow), areas of glomerular basement membrane duplications forming double contours (double-headed arrows), and increased cellularity (asterisk shows location of monocytes and/or endothelial cells). (**b**) These pathologic lesions were less prominent in 9 month-old *fH*−/−/*μMT* mice. There are relatively small sub-endothelial deposits (arrowheads), otherwise the glomerulus has normal ultra-structure. Original magnification × 3000. Bar = 2 μm.
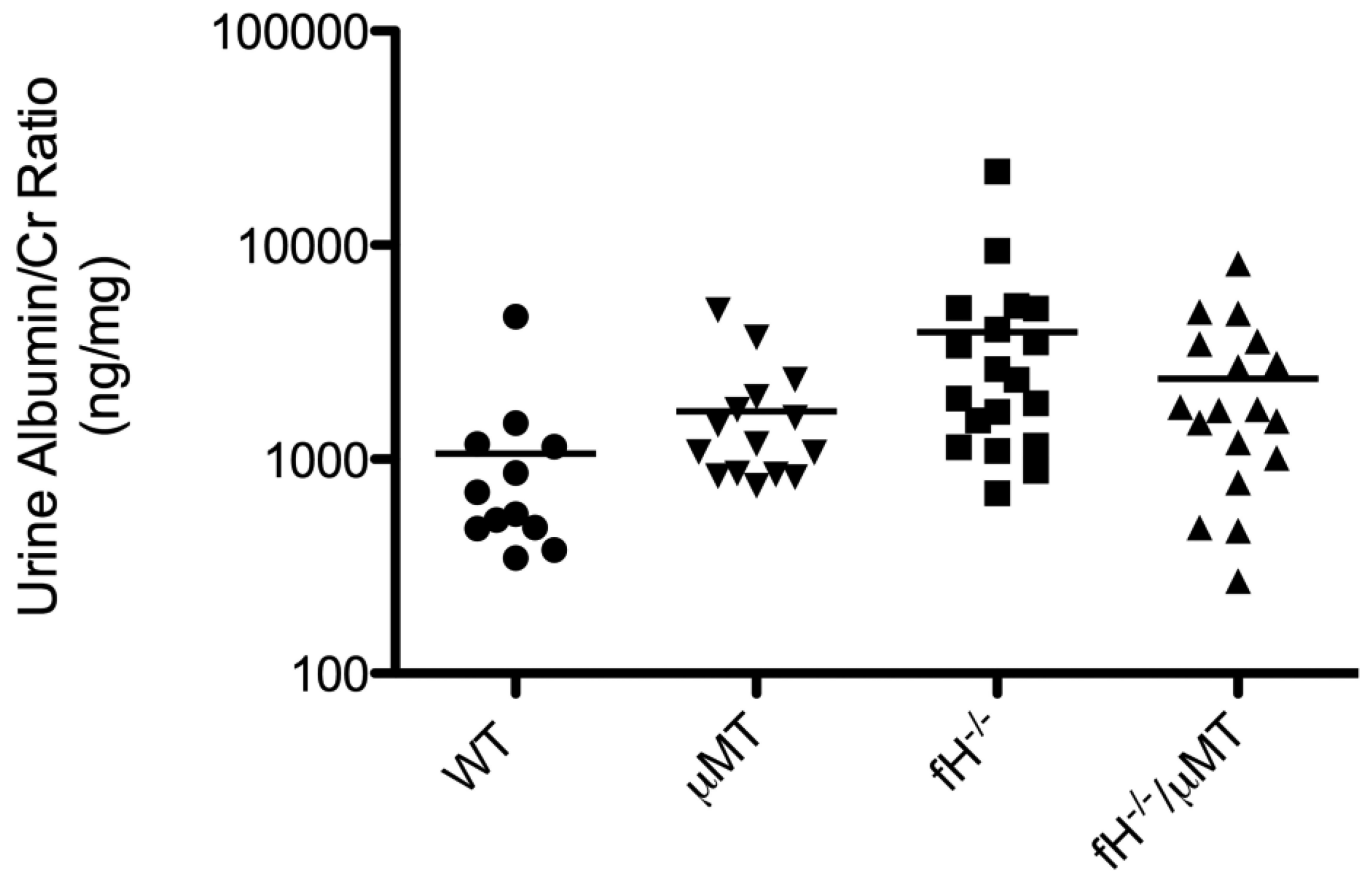
**Figure 11. B cells contribute to the development of albuminuria in factor H deficient mice**
Urine albumin/creatinine levels were determined in 9 month-old animals. There was a trend towards greater albuminuria in *fH*$^{-/-}$ over wild-type (WT) mice. Albuminuria was attenuated in the *fH*$^{-/-}$/*μMT* group but did not achieve statistical significance. Individual data points are presented. Lines represent means.