Chimeric maternal cells in offspring do not respond to renal injury, inflammatory or repair signals

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Abbreviations: EGFP, enhanced green fluorescent protein; MHC, major histocompatibility complex; MMc, maternal microchimerism; FMc, fetal microchimerism; UUO, unilateral ureteral obstruction; RT-qPCR, real-time quantitative PCR; gDNA, genomic DNA; gEq, genome equivalents; BUN, blood urea nitrogen

Maternal microchimerism (MMc) can persist for years in a child, and has been implicated in the pathogenesis of chronic inflammatory autoimmune diseases. Chimeric cells may either contribute to disease by acting as immune targets or expand in response to signals of injury, inflammation or repair. We investigated the role of maternal cells in tissue injury in the absence of autoimmunity by quantifying MMc by quantitative PCR in acute and chronic models of renal injury: (1) reversible acute renal injury, inflammation and regeneration induced by rhabdomyolysis and (2) chronic injury leading to fibrosis after unilateral ureteral obstruction. We found that MMc is common in the mouse kidney. In mice congenic with their mothers neither acute nor chronic renal injury with fibrosis influenced the levels or prevalence of MMc. Maternal cells expressing MHC antigens not shared by offspring (H2^{b/d}) were detected at lower levels in all groups of homozygous H2^{b/b} or H2^{d/d} offspring, with or without renal injury, suggesting that partial tolerance to low levels of alloantigens may regulate the homeostatic levels of maternal cells within tissues. Maternal cells homozygous for H2^b were lost in H2^{b/d} offspring only after acute renal failure, suggesting that an inflammatory stimulus led to loss of tolerance to homozygous maternal cells. The study suggests that elevated MMc previously found in association with human autoimmune diseases may not be a response to non-specific injury or inflammatory signals, but rather a primary event integral to the pathogenesis of autoimmunity.

Introduction

The bidirectional passage of cells during pregnancy leads to persistent maternal and fetal microchimerism (MMc, FMc).^{1,2} Both have been implicated in the pathogenesis of idiopathic autoimmune diseases that resemble graft-versus-host disease after stem cell transplant.³⁻⁵ We have documented MMc and FMc in organs that are often targeted in autoimmunity, raising the possibility that chimeric cells could act as targets for the host immune system, stimulating chronic inflammation.⁶⁻⁸ Although maternal and fetal cells have been found at increased levels in patients with autoimmune disease, it is not known whether or not this is a primary event, whereby the higher numbers of chimeric cells directly contribute to disease. Chimeric cells could instead be responding to non-specific stimuli of chronic inflammation and tissue injury, and not involved in the autoimmune process at all (Fig. 1).^{9,10}

During acute renal injury tubular damage and inflammation can lead to renal failure with the potential for regeneration and recovery of normal renal function.^{11,12} However, even patients who fully recover can have poor long-term prognoses and chronic renal failure for unknown reasons.^{11,13} In animal models, transplanted bone marrow-derived stem cells can migrate to the kidney in response to renal injury, though whether bone marrow-derived chimeric stem cells are helpful or harmful to the recovery process and how they affect long-term renal function is controversial.^{14,15} Even less is known about the potential for non-host stem cells, naturally derived during pregnancy (MMc and FMc), to contribute to renal injury or regeneration. Maternal cells could respond to injury signals in the kidney just as autologous stem cells are called from the bone marrow.¹⁶ Maternal cells, however, would carry foreign, allogeneic genes and thus have the potential to express immunostimulatory proteins. Inflammatory signals that increase expression of histocompatibility molecules could

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Figure 1. Model for the role of MMc in chronic renal injury. Maternal cells are normally present at low levels in healthy tissues. Signals released from injured tissues (1), attract inflammatory cells (2), in turn inducing release of G-CSF and other cytokines that recruit stem cells from bone marrow to emerge in the peripheral blood and then migrate to the tissues (3). Maternal cells residing in the bone marrow are recruited alongside host stem cells. Maternal cells participate in tissue repair (4), leading to differentiated chimerism in the parenchyma with the capacity to express allogeneic proteins (5). Maternal antigens, in higher levels due to increased cell numbers and inflammation-induced expression, then continue to stimulate host lymphocytes, leading to tolerance or to chronic inflammation similar to a chronic infection. This model explains how persistent maternal cells could both contribute to and result from tissue injury and chronic inflammation.

make the maternal cells more visible to the host immune system, thereby breaking the normal tolerance to maternal antigens.

To dissect the association of MMc in autoimmune diseases from that of non-specific inflammation and fibrosis, we studied MMc in two different models of renal injury. In the rhabdomyolysis model, acute myoglobinemia leads to bilateral renal tubular injury. The kidneys then recover and fully regenerate, returning to normal function.¹⁷ The rhabdomyolysis model is thus analogous to the acute renal injury of critically ill patients, i.e., trauma. In the unilateral ureteral obstruction (UUO) model, one kidney is injured, the insult is not removed, and the chronic injury leads to fibrosis through well-characterized mechanisms, thereby mimicking chronic inflammatory disease, such as systemic sclerosis, glomerulosclerosis or pulmonary fibrosis.^{18,19} In either model, changes in tubular cell proliferation and apoptosis occur in concert with interstitial cell apoptosis and proliferation, leading to interstitial damage.²⁰ Molecules with danger-associated molecular patterns released from injured tissue trigger an infiltration of macrophages that further promote the progression of kidney fibrosis and damage (reviewed in refs. 21 and 22). We hypothesized that maternal cells could expand or be recruited in response to the signals of acute or chronic injury and consequently exacerbate damage to the kidney.

We found that MMc was not influenced by renal injury if the mothers were congenic with their offspring. However, maternal cells homozygous for MHC alleles that populated kidneys from heterozygous, haploidentical offspring were lost after acute renal

failure, suggesting that local inflammatory signals may activate host immunity to foreign antigens. The results do not support a model in which maternal cells respond to non-specific inflammatory signals, but rather indirectly support a primary role for maternal cells as alloreactive targets in idiopathic inflammatory disease.

Results

Acute renal injury induced by rhabdomyolysis. Our initial experiments sought to address how congenic maternal chimeric cells respond to injury and inflammatory signals. Rhabdomyolysis was induced in 43 mice by intramuscular injection of glycerol, as previously described in reference 17. This model allowed for the investigation of MMc in kidneys harvested at distinct stages of acute injury (days 1–3), inflammation (days 4–7) and regeneration (days 14–28). Control mice were either left untreated or given intramuscular injections of saline. Glycerol treatment led to renal failure in 63% of animals, as indicated by at least a two-fold increase in blood urea nitrogen (BUN) levels one to three days after surgery (Sup. Fig. 1A). In comparison, only 7% of saline-treated and none of the five untreated controls had evidence of renal failure. At day seven, BUN levels began to decrease in all mice, reaching normal levels by day 14. Tubular injury in glycerol-treated animals was confirmed by histology of kidney sections, with an inflammatory infiltrate and tubular injury evident by day three and pyknotic nuclei, apoptotic cells and tubular casts evident by day seven (Sup. Fig. 1B). Tubules were fully regenerated and appeared histologically normal by day 28.

Phenotypes of maternal cells in kidneys did not correlate with disease. By fluorescence microscopy we found no differences in phenotypes of maternal cells in either model or controls (Sup. Fig. 2). Though the GFP expression vector with β -actin promoter and CMV enhancer was developed for ubiquitous expression, we detected GFP by immunofluorescence in only 30–60% of renal cells. EGFP expression was found most readily in cortical areas within tubules, with lower levels detected in glomeruli. Thus, estimates of MMc levels by microscopy are lower than actual levels.

We found that most of the GFP⁺ chimeric maternal cells expressed CD45, and thus were hematopoietic. Maternal cells expressing CD45 were found in two of three kidneys at levels estimated at 3.1 and 12.3 maternal cells/million host cells. In contrast, lower numbers of maternal cells bound *Lotus tetragonolobus* lectin, a marker for proximal tubular cells (two of four kidneys assayed at levels of 0.8 and 2.96/million host renal cells). Maternal endothelial cells represented even fewer of the chimeric maternal cells (1.4/million host cells). Each marker could only be used in one tissue section, precluding the detection of cells that may express more than one of these markers and preventing precise comparisons between fractions of maternal cells with each phenotype within one area. This data demonstrates that maternal cells can migrate to the kidney and take the form of hematopoietic, tubular or endothelial cells, with no influence from injury, inflammation or regenerative signals.

Quantification of MMc in the kidney after rhabdomyolysis. A sensitive assay was required to detect and quantify low levels of MMc in genomic DNA (gDNA) isolated from the kidney. MMc was assayed in enhanced green fluorescent protein (EGFP)^{-/-} mice born to EGFP^{+/-} mothers by Real-Time quantitative PCR (RT-qPCR), targeting the non-inherited maternal EGFP sequence. An assay was developed for the EGFP sequence with a sensitivity of one genome equivalent (gEq) of EGFP^{+/-} DNA in 100,000 gEq EGFP^{-/-} host DNA (Sup. Fig. 3). At least 100,000 gEq of genomic DNA isolated from each kidney was assayed, as determined by RT-qPCR of the two-copy Apolipoprotein B (*ApoB*) locus.

MMc was detected in the majority of kidneys (Fig. 2). We found no change in the level of MMc in response to acute renal injury, inflammation or regeneration. The trend toward an increase in the level of MMc seven days after injury was not significant. The prevalence of MMc was, however, increased in mice with more severe renal failure. Over all time points, MMc was detected in 82% of mice with acute renal injury versus 55% of saline-treated mice (Fig. 2B and OR 6.1, 95% confidence interval 1.5–24, $p = 0.014$). MMc was detected in all mice with BUN levels >125 mg/dL (Fig. 2C). The significance of this finding, however, is not clear, as MMc was also detected in 100% of untreated mice, and so may be an artifact of small sample groups.

The mice varied in the severity of renal injury, as reflected by a range of BUN detected (Fig. 2C). Because higher levels of MMc could break the threshold for lymphocyte tolerance in the offspring, and so cause increased inflammation and injury triggered by the expression of foreign maternal EGFP molecules, we tested the association between MMc levels and severity of disease. We found no correlation between MMc and the maximum BUN level (Fig. 2C). Likewise, there was no difference in BUN levels at any time point in animals with MMc compared to those without detectable MMc. Therefore, chimeric maternal cells were not recruited or expanded in the kidney in response to non-specific signals of acute injury, inflammation or regeneration.

Chronic renal fibrosis was not associated with MMc in the kidney. Previous studies have reported increased FMc or MMc in patients with fibrotic autoimmune diseases like scleroderma.^{1,23} In most autoimmune diseases, organ fibrosis is a final stage of chronic inflammation. To test the response of MMc to chronic injury resulting in fibrosis, we induced renal fibrosis by UUO in eleven mice congenic with their mothers except for the maternal EGFP gene, as previously described in reference 18 and 19. Kidneys were harvested after 14 or 30 days. We used the contralateral kidneys as controls, which reduced the inter-mouse variability in MMc levels and increased the statistical power to detect differences between injured and control kidneys. We found the macrophage infiltration and severity of interstitial fibrosis in the kidneys of our UUO mice to be consistent with previous observations (data not shown).^{18,19} By 14 days, collagen accumulation in the extracellular matrix was observed and persisted through at least 30 days. The architecture of contralateral control kidneys remained normal.

Figure 2. MMc did not respond to acute renal injury. After induction of rhabdomyolysis with glycerol (G) or treatment of control mice with saline (S), MMc was quantified by real time qPCR amplification of the non-inherited maternal EGFP gene in gDNA extracted from kidneys. No Tx, no treatment (mice were not injected). (A) MMc/ml, gEq of maternal gDNA per million gEq of total kidney gDNA. Bars represent median values. (B) Percent of mice with chimerism in each treatment group. Glycerol vs. saline, OR 3.8, 95% confidence interval 0.8–18.7, $p = 0.05$. Glycerol vs. no treatment and saline vs. no treatment, $p > 0.05$. (C) The severity of renal failure, as indicated by the maximum BUN level (mg/dL) for each mouse during the treatment course, did not correlate with the levels of MMc. Data was pooled for mice assayed on all days.

UUO had no effect on the level of MMc in the kidney, as measured by maternal GFP sequences. The level of MMc was similar in obstructed kidneys, contralateral control kidneys and untreated kidneys at days 14 and 30 (Fig. 3A). Overall MMc

was detected in 73% of obstructed kidneys and 73% of contralateral control kidneys (Fig. 3B), demonstrating no change in prevalence.

Renal atrophy and contralateral hypertrophy after UUO. Chronic UUO impairs the growth of the obstructed kidney and stimulates compensatory growth in the contralateral control kidney.^{22,24} Consistent with these reports, we found a significant decrease in the weight of obstructed kidneys compared to contralateral controls or untreated control kidneys at 7, 14 and 30 days after surgery (Sup. Fig. 4A; $p < 0.05$). The contralateral control kidneys weighed more than the untreated control kidneys at all time points, demonstrating their compensatory growth.

We hypothesized that maternal cells could participate in either the fibrosis of the affected kidney or the hypertrophy of the contralateral compensating kidney. We did not, however, detect any correlation between the level of MMc and the weight of the kidneys (Sup. Fig. 4B). Therefore, chimeric maternal cells did not respond to signals of chronic injury or fibrosis, nor did they respond to hypertrophic signals in contralateral kidneys.

Taken together, the results thus far demonstrated that chimeric maternal cells congenic to their offspring did not respond to signals of acute injury, inflammation, repair, chronic injury or fibrosis, all processes that occur in chronic autoimmune diseases.

Allogeneic maternal cells are eliminated after renal injury. The model for how MMc could contribute to chronic renal injury implies that maternal antigens perpetually activate host lymphocytes (Fig. 1). To address whether allogenes carried by chimeric maternal cells impact the persistence of MMc and/or the severity of renal failure, we induced rhabdomyolysis or UUO in C57BL/6 x Balb/c F1 ($H-2^{b/d}$) and F2 ($H-2^b$ or $H-2^d$) mice that differed from their mothers at the $H-2$ loci (Fig. 4A). We found that the level of MMc significantly decreased in the F1 $H-2^{b/d}$ mice born to $H2^{b/b}$ mothers after acute renal failure, as compared to $H-2^{b/b}$ mice genetically identical to their mothers ($p = 0.0003$) or to F2 $H2^{b/b}$ or d/d mice born to $H2^{b/d}$ mothers ($p = 0.036$, Fig. 4B).

In the UUO model of chronic inflammation we found that F2 ($H2^{b/b}$ or $H2^{d/d}$) mice born to $H2^{b/d}$ mothers had significantly reduced levels of MMc in both the UUO and contralateral control kidneys as compared to those of the $H-2^{b/b}$ mice that were genetically identical to their mothers (Fig. 4C; $p = 0.0053$). Though there was no relationship between overall weight of the kidneys and MMc (Sup. Fig. 4B), suggesting that the level of injury or compensatory hypertrophy did not involve expansion of maternal cells, we did detect a significant increase in the weight of the hypertrophied contralateral kidneys in the homozygous $H2^{b/b}$ or $H2^{d/d}$ pups born to heterozygous $H2^{b/d}$ mothers (Sup. Fig. 4C). It is possible that a systemic inflammatory response contributed to greater hypertrophy, but this possibility was not directly tested.

This data suggests that non-inherited maternal proteins encoded by $H2^d$ or $H2^b$ may be recognized by cytolytic lymphocytes in $H2^{d/d}$ or $H2^{b/b}$ offspring, allowing for normal regulation

of the level of MMc in the kidney. Homozygous maternal cells, on the other hand, are tolerated by the host immune system until acute injury occurs, when tolerance is broken and maternal cells are depleted.

Discussion

MMc and FMc have been implicated in the etiology of autoimmune diseases for over a decade now, but why levels of chimeric cells would be elevated in blood or tissues of patients is not understood.^{2,6,7,23,25} Maternal cells could be transferred in higher numbers during the pregnancy, secondary to pregnancy complications or to homing signals like VEGF.^{26,27} Alternatively, maternal cells may have increased survival, proliferative capacity, or homing to target organs during homeostatic or inflammatory conditions. Chronic inflammation appearing to be autoimmune has been hypothesized instead to be alloimmune, with chimeric cells responding to non-shared host antigens or host lymphocytes responding to non-shared maternal antigens.⁴ In healthy animals tissue injury induces cytokine expression to stimulate bone marrow-derived stem cells to emerge in the blood, migrate into injured tissues, and participate in tissue regeneration.²⁸⁻³¹ As autoimmune disease causes inflammation-mediated tissue injury, we hypothesized that elevated levels of MMc found in patients could be secondary to non-specific signals of inflammation or tissue injury, and thus a result, rather than a cause of chronic inflammation. We found in two distinct models of renal injury that maternal cells are not increased (either by expansion or recruitment) in response to tissue injury, inflammation, regeneration or fibrosis. Neither rhabdomyolysis, a systemic disease resulting in acute renal failure, nor UUO, a chronic disease model of fibrosis, was associated with increased MMc in the kidney.

If maternal cells are not expanded secondary to non-specific inflammatory signals, they could instead be contributing to the chronic inflammation associated with autoimmune disease, and with other chronic inflammatory conditions such as atherosclerosis and Type I diabetes. However, we found no association between levels of MMc and markers of severity of renal failure. Thus, at least in the injury models tested, elevated levels of MMc do not appear to exacerbate inflammation-mediated tissue injury. There was variation in the levels of MMc between mice in each group, but the models did not allow us to determine if animals who started out with higher levels of MMc then went on to develop more severe inflammation-mediated injury or fibrosis.

Autoimmune diseases are usually diagnosed after a prolonged period of inflammation and tissue injury. The mouse models employed may be too short-lived to accurately reflect events occurring in chronic autoimmune disease. Moreover, the inflammatory or injury signals may not be the same in autoimmunity, involving different tissues, lymphocyte subsets activated and cytokines. For instance, maternal T cells have been implicated in juvenile dermatomyositis,³² whereas T lymphocytes may not be important in the pathogenesis of rhabdomyolysis and monocytes are more important in renal fibrosis.

MHC alleles have been found previously to be important in tolerance to maternal cells in part through induction of

Figure 3. Maternal cells did not expand after chronic inflammation and fibrosis. Left ureters were obstructed to cause renal fibrosis with onset between 14 and 30 days (UUO). Contralateral (right) kidneys served as controls for each mouse (Contra). MMc was quantified by real time qPCR amplification of the non-inherited maternal EGFP gene in gDNA extracted from kidneys. (A) MMc/mil, gEq of maternal gDNA per million gEq of total kidney gDNA. No Tx, no treatment control mice. (B) percent of mice with chimerism in each treatment group.

regulatory T cells during pregnancy which are then maintained through nursing.³³⁻³⁵ Minor histocompatibility alleles may be even more important.^{36,37} After we established that maternal cells did not expand during inflammation, we next hypothesized that induction of inflammatory signals might break tolerance to non-inherited maternal antigens (NIMA). We found that maternal cells were *eliminated* after acute renal failure, but only homozygous H2^{b/b} maternal cells in heterozygous H2^{b/d} offspring. One explanation for this finding could be the activation of progeny NK cells by the initial inflammatory stimulus. H2^{b/d} NK cells are normally inhibited by self H2^b and H2^d molecules. As maternal H2^{b/b} cells would not express protective H2^d they would be targeted by the host NK cells, and thus depleted from the affected tissues.

We also detected a decrease in maternal H2^{b/d} cells in the kidneys of H2^{b/b} or H2^{d/d} mice. Though apparent in all mice, the difference was most significant after UUO, in both affected and unaffected kidneys, suggesting an entirely different mechanism for maintenance of heterozygous MMc in which systemic

inflammatory signals induce expression of MHC molecules on maternal cells throughout all organs to high enough levels that they are now recognized by the host cytolytic T cells, which expand in population and then lyse maternal cells in both affected and unaffected kidneys. Such a systemic response would be expected to deplete MMc from other organs as well, with unknown consequences. Autoimmune diseases are known to initiate or flare after infection, suggesting an inflammatory stimulus breaks tolerance. Direct links between host regulatory T cells, NK cells and cytolytic T cells responding to NIMA after inflammatory triggers could be tested in models of tissue injury.

Although we interpret the data as a reflection of the influence of MHC alleles on maternal cell survival, the levels of maternal cells may instead represent differential transfer of maternal cells during pregnancy or proliferative capacity related to non-MHC genes carried by the Balb/C versus B6 mice. Mice congenic for all alleles except H2 could be used to explore this possibility. Previous studies quantifying MMc in mice of different MHC haplotypes have not assayed MMc in the kidney, but do report small but statistically significant decreases in MMc in homozygous mice with heterozygous mothers, under homeostatic conditions.³⁸ In other tissues, levels of MMc correlated with tolerance to NIMA, implying loss of tolerance in mice in which MMc was not detected.^{35,36}

We detected MMc expressing EGFP in the kidney by immunofluorescence in the form of renal tubular cells, glomerular endothelial cells and hematopoietic cells. The possibility exists that what appear to be chimeric tissue-specific differentiated cells may actually be host cells that have fused with hematopoietic maternal cells or taken up EGFP from maternal hematopoietic cells. Donor bone marrow-derived stem cell fusion with tubular epithelial cells has been reported at a frequency of 7/10,000 tubular cells,³⁹ though may increase to 50% in severe injury, especially if they have a selective advantage.⁴⁰ Maternal cells fused with host renal tubular cells may still contain functional maternal gDNA, and thus express allogeneic non-inherited maternal proteins, so the origin of maternal cells may not be relevant to alloimmunity.⁴¹

In summary, this report refutes the hypothesis that MMc is elevated in autoimmune diseases as a response to inflammation and supports a model where allogeneic maternal cells are recognized and targeted by the host immune system during any inflammatory response.

Materials and Methods

Animals. All procedures were performed in accordance with the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of Seattle Children's Research Institute approved the study protocol. Balb/C and transgenic Tg(CAG-EGFP)B5Nagy/J mice expressing EGFP under the regulation of the chicken beta-actin promoter and CMV intermediate early enhancer were purchased from the Jackson Laboratory. This mouse is on the B6 background, and was formerly marketed under a different name: Tg(CATB-EGFP)B5Nagy/J. EGFP^{+/+}

Figure 4. MHC compatibility determines the survival of maternal cells in the kidney. (A) The breeding scheme for testing the effects of MHC H2 alleles on MMc after renal injury. Boxes surround offspring tested. MMc/mil, gEq of maternal gDNA per million gEq of total gDNA kidney. (B) MMc levels in kidneys after rhabdomyolysis. (C) MMc levels in the kidneys after ureteral obstruction and in hypertrophied contralateral control kidneys.

females were crossed with C57BL/6J (EGFP^{-/-}) males to generate EGFP^{+/-} F1 females, which were back-crossed to the C57BL/6J (EGFP^{-/-}) males so that maternal EGFP^{+/-} cells could be detected in EGFP^{-/-} pups. Mice heterozygous for the H2 loci were obtained by breeding Tg(CAG-EGFP)B5Nagy/J GFP^{+/-} H2^{b/b} females to Balb/C H2^{d/d} GFP^{-/-} males, and MMc was assayed in GFP^{-/-} H2^{b/d} offspring (F1, Fig. 4A). The GFP^{+/-} H2^{b/d} mice from this F1 generation were then bred to GFP^{-/-} H2^{b/d} mice to produce the F2 generation so that maternal GFP sequences could be detected in homozygous GFP^{-/-} H2^{d/d} and H2^{b/b} offspring.

Renal injury models. Rhabdomyolysis was induced in six-week-old EGFP^{-/-} mice born to EGFP^{+/-} mothers by intramuscular injection of 50% glycerol (8.5 μ L/gram of body weight) (Balb/C mice required 60% glycerol), or an equal volume of normal saline, as previously described in reference 17. UUO was carried out as previously described in reference 18 and 19. Briefly, the left ureter was tied in two places: one ligature close to the renal pelvis and the second approximately 2 mm distal to the first.

Blood urea nitrogen (BUN) assay. Plasma BUN levels were determined using the plasma protocol for a commercially available kit (Bioassay Systems). For mice treated with glycerol, only those with renal failure, defined as a two-fold increase in BUN over baseline, were included in the analysis. Saline-treated mice with abnormal renal pathology were excluded from the analysis.

Tissue collection and preparation. Mice from the two renal injury models were sacrificed at days 3, 7, 14 or 28 for rhabdomyolysis and days 3, 7, 14 or 30 for UUO by cardiac puncture exsanguination followed by direct cardiac perfusion with anticoagulant citrate dextrose (2.45% dextrose, 2.2% sodium citrate, 0.73% citric acid). Both kidneys were harvested, the capsules immediately removed, and the kidneys sectioned sagittally in order to include both cortex and medulla. Preliminary studies suggested that background fluorescence could be decreased by perfusion in situ, in order to wash out red blood cells. In addition, we compared methods of tissue preservation to optimize GFP fluorescence, and determined that fixation in 4% paraformaldehyde/PBS for 2 hours, embedding in Tissue-Tek OCT (Sakura Finetek), and snap-freezing gave the best results. Two diagonally opposed quarters of each kidney were designated for DNA extraction, flash frozen and stored at -80°C.

DNA extraction and real-time quantitative PCR for the EGFP transgene. Kidney sections were digested at 55°C overnight with RNA grade Proteinase K (Invitrogen). DNA was extracted using the Puregene DNA Purification Kit (Gentra Systems) according to the manufacturer's protocol for 50–100 mg of frozen solid tissue.

To identify EGFP^{+/-} and EGFP^{-/-} mice, genotyping was performed by PCR amplification of genomic DNA isolated via DirectPCR Lysis (Viagen Biotech) (Sup. Fig. 3). PCR primer sequences and protocols were derived from Jackson Laboratory published protocols. The EGFP transgene primers, IMR0872 (5'-AAG TTC ATC TGC ACC ACC G-3') and IMR1416 (5'-TCC TTG AAG AAG ATG GTG CG-3'), amplified a 173 bp fragment. The IL-2 gene was used as an internal control. The primers IMR0042 (5'-CTA GGC CAC AGA ATT GAA

AGA TCT-3') and IMR0043 (5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3') amplified a 324 bp fragment of the IL-2 sequence. Reactions were performed using GoTaq Green Master Mix (Promega) and the amplification conditions used were as follow: 94°C for 1.5 min, 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min each for 35 cycles; final extension was performed at 72°C for 2 min.

To quantitate the chimeric maternal EGFP transgene in genomic DNA (gDNA) from EGFP^{-/-} progeny, RT-qPCR amplification was performed using the following primers: forward primer 5'-AGC AGC ACG ACT TCT TC-3'; reverse primer 5'-TCG TCC TTG AAG AAG ATG-3'; probe 5'-FAM-AGT CCG CCA TGC CCG AAG GCT-BHQ1-3' (Integrated DNA Technologies). The amplification product corresponded to a 73 bp sequence on the EGFP transgene. To quantify host gDNA, *Apob* was amplified on the same plates using the following reagents: forward primer 5'-TGC CCA TTT CCT TTC TTC-3'; reverse primer 5'-CAG ACG TGA TGT TCC CT-3'; probe 5'-FAM-CTT CAT TTG TCA CTG ACG CCC TGC-BHQ1-3'. The *Apob* amplification product was a 73 bp sequence. Each PCR reaction consisted of 25 μ L Sensimix dT (Bioline), 300 nM each primer, 100 nM dual-labeled probe, and approximately 20,000 genome equivalents (gEq, 125 ng) of target kidney gDNA. The amplification conditions were 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds and 55°C for 45 seconds, using the Applied Biosystems 7500 Real-Time Thermocycler System. Calibration curves were constructed with EGFP^{+/-} gDNA mice diluted in 20,000 gEq GFP^{-/-} gDNA. The standard curve was linear between one and 500 gEq of EGFP in 20,000 gEq host DNA in each well. To ensure the sensitivity of our assay, all standard curves were required to have an R² value ≥ 0.99 . Sensitivity was increased by assaying multiple wells for each sample to total at least 100,000 gEq per sample. Total gDNA was estimated using a standard curve of commercially-available mouse gDNA (Promega).

Histology and immunofluorescence analysis. Renal injury was confirmed by hematoxylin and eosin staining. For immunofluorescence, frozen kidney sections were washed with PBS/0.05% Tween-20 and blocked in 1% normal horse serum in PBS (Vector Elite Vectastain ABC Kit, Vector Laboratories). The following primary reagents were used: rabbit anti-EGFP (Novus Biologicals); biotinylated rat anti-CD45 (BD Pharmingen); rat anti-CD31 (eBiosciences); biotinylated Lotus Tetragonolobus lectin (Vector Laboratories). Sections were incubated for 1 hour with primary reagent in 1x PBS/0.03% Tween-20/1% Bovine Serum Albumin, washed with PBS/0.05% Tween-20, and blocked again in 5% normal goat serum (Jackson Immunology). The sections were then incubated with a 1/100 dilution of fluorescein-labeled goat anti-rabbit IgG (Vector Laboratories), Alexa Fluor 568 Streptavidin, or Alexa Fluor 568 goat anti-rat IgG (Invitrogen) to detect specific antigens. Sections were washed a final time in PBS/0.05% Tween-20 followed by a one minute incubation in DAPI (1.0 μ g/mL in PBS) to identify nuclei. Slides were mounted with VectaShield HardSet Mounting Medium (Vector Laboratories). Autofluorescence was distinguished from true green fluorescence with a filter for yellow, in which all tissues

demonstrate background fluorescence. No cells expressing EGFP were found in negative control kidneys from EGFP^{-/-} animals born to EGFP^{-/-} parents, run with each experiment. To determine the frequency of MMc in the kidney, four images from each of four kidney sections were analyzed with the Image-Pro Plus software (Media Cybernetics). The density of cells was determined by counting DAPI-stained nuclei per surface area (mm²).

Statistical analyses. Significance between levels of MMc in mice with renal failure and controls was tested with the two-sample Wilcoxon Rank Sum (Mann-Whitney) test. Differences between pairs of kidneys in UUO mice were compared using the Student's t test. Prevalence differences between groups were tested using the Fisher's Exact Test.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/chimerism/article/16446

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