Fate mapping reveals compartment-specific clonal expansion of mononuclear phagocytes during kidney disease

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Kidney International (2023) **104,** 605–610; https://doi.org/10.1016/j.kint.2023.04.031

KEYWORDS: chronic kidney disease; glomerulonephritis; macrophages

Renal mononuclear phagocytes (MNPs), including macrophages and dendritic cells, form a contiguous sentinel network throughout the kidney interstitium and participate in maintaining homeostasis and the defense against pathogens.[1,2] MNPs are also involved in inflammatory kidney disease (e.g., in crescentic glomerulonephritis and inflammasome-mediated tissue fibrosis).[1] Accumulation of MNPs is a prominent feature in nephritis,[3] and reducing MNP numbers is protective in most nephritis models.[3] The recruitment of circulating monocytes contributes to MNP accumulation, and its inhibition (e.g., by blocking chemokines or their receptors) ameliorates nephritis partially.[2] In addition, local proliferation of tissue-resident MNPs might theoretically contribute to their accumulation in nephritis. This would impact therapeutic approaches to treat nephritis by preventing the recruitment of MNP precursors.

Most reporter mice used for studying MNPs express a single fluorescence molecule in the whole MNP population and, hence, cannot discriminate individual cells. Such discrimination has become possible with Confetti multicolor fluorescence reporter mice that stochastically express different fluorochromes in distinct cell types after crossing to suitable mice transgenically expressing CRE recombinase. In nephrology, such mice have been used to differentially label kidney-resident cells, like podocytes, tubular epithelial cells, or renin-lineage cells.[4] For motile cells, such as renal MNPs, this technique also allows distinguishing between recruitment and local proliferation, because the former results in homogenic distribution of same-color MNPs, whereas local proliferation generates clones of the same fluorescence color. A suitable tool is the Microfetti mouse line, generated by crossing Confetti mice with CX_3CR1-CRE mice, in which microglia cells randomly expressed 1 of 4 fluorescent proteins: green fluorescent protein (GFP), yellow fluorescent protein, red fluorescent protein, and cyan fluorescent protein.[5,6] We previously showed that also pathogenic renal MNPs in nephrotoxic nephritis (NTN) express and depend on CX_3CR1.[7] Thus, Microfetti mice allowed clarifying whether these MNPs locally proliferate in nephritis models.

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Received 8 August 2022; revised 14 April 2023; accepted 27 April 2023; published online 7 June 2023

RESULTS

Confetti-labeled MNPs in kidneys of Microfetti mice

To label renal MNPs, a single dose of tamoxifen was injected s.c. into Microfetti mice, as described[5,6] (Figure 1a and Supplementary Methods). One week later, Confetti-labeled cells were evident in the renal cortex and medulla (Figure 1b; Supplementary Figure S1). Costaining with F4/80 confirmed that Confetti^{+} cells were MNPs (Figure 1a and b). Each individual Confetti^{+} MNP expressed 1 of the 4 fluorescent proteins in different cellular compartments: GFP in the nucleus, cyan fluorescent protein in the cell membrane, yellow fluorescent protein in the cytoplasm, and red fluorescent protein in the cytoplasm (Supplementary Figure S2A and B). No labeling was seen in Microfetti mice not injected with tamoxifen, and proliferating MNPs were not preferentially labeled (data not shown), consistent with previous

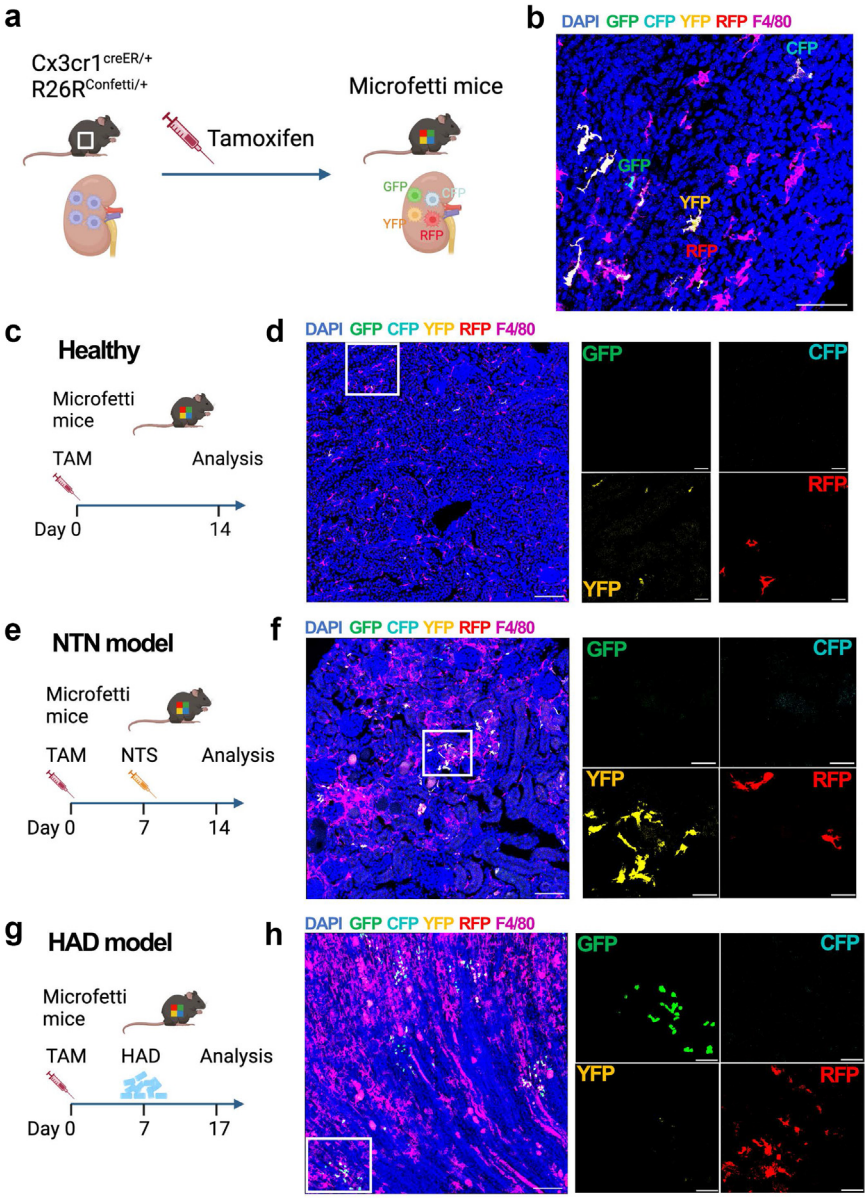

Figure 1 | Differentially labeled mononuclear phagocytes (MNPs) in kidneys of Microfetti mice. (**a**) Scheme of induction of Confetti^{+} renal MNPs in Microfetti mice. (**b**) Representative maximum-intensity projection of a confocal stack from the kidney of a Microfetti mouse with green fluorescent protein (GFP) in the nucleus (green), yellow fluorescent protein (YFP) in the cytoplasm (yellow), red fluorescent protein (RFP) in the cytoplasm (red), cyan fluorescent protein (CFP) in the cell membrane (cyan), F4/80-immunolabeled MNPs (magenta), and 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (blue). Bar = 50 μm. (**c,e,g**) Diagram of analysis of healthy Microfetti mice (**c**), and Microfetti mice with nephrotoxic nephritis (NTN) (**e**) and high adenine diet (HAD) (**g**). (**d,f,h**) Representative maximum-intensity projection of a confocal stack from the kidney of healthy (**d**), NTN (**f**), and HAD (**h**) Microfetti mice. Confetti channels of the rectangle in the left panel are shown in the right panel. Bar = 100 μm (left panel) and 20 μm (right panel). NTS, nephrotoxic serum; TAM, tamoxifen. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

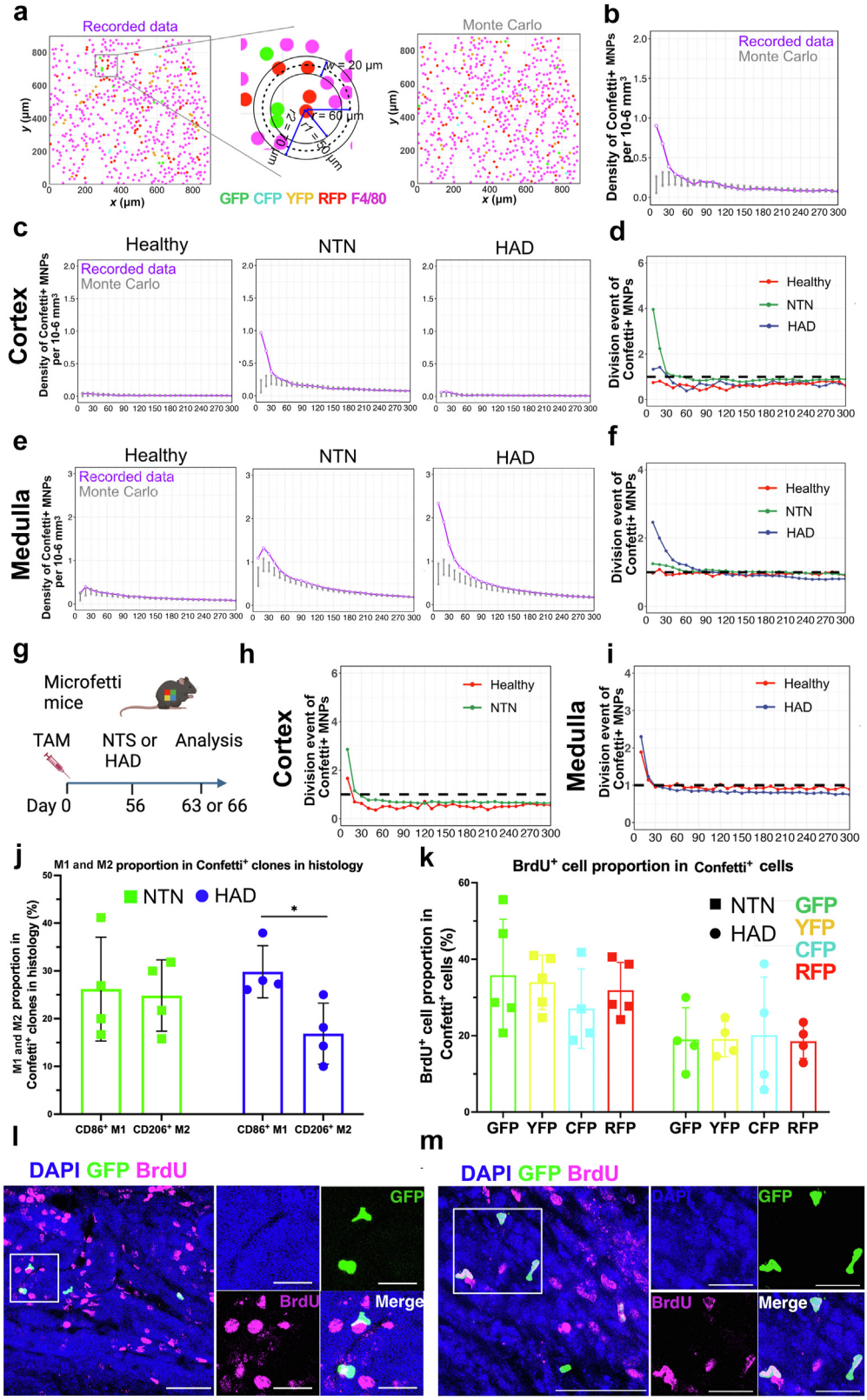

Figure 2 | Clonal intrarenal expansion of mononuclear phagocytes (MNPs) in nephrotoxic nephritis (NTN) and high adenine diet (HAD). (**a**) Strategy for analysis of distribution of renal Confetti$^+$ MNPs. For each individual Confetti$^+$ MNP in a confocal stack (left), the density in concentric rings of 20 µm width (w) and radii (r) from 10 to 300 µm was determined, here illustrated for 60-µm radius as an example (middle). Monte Carlo (MC) simulation (right) of random distribution of renal Confetti$^+$ MNPs was performed 100 times to calculate the baseline density for MNPs that would result randomly. (**b**) Example of the MNP density (*y* axis) calculated by dividing corresponding same-color Confetti MNP counts by the ring volume of each ring size (*x* axis). The data density is shown in purple, and that of MC-simulated data is shown in (continued)

findings.[6] This indicated that stochastic multicolor labeling of renal MNPs was possible by using Microfetti mice.

To study clonotypic labeling in models of glomerulonephritis and tubulointerstitial nephritis, we next labeled MNP in mice with NTN[7] or under high adenine diet (HAD),[8] respectively. Healthy Microfetti mice were used as a control and showed a homogenic distribution of Confetti colors (Figure 1c and d). One week after NTN or 10 days after HAD induction, kidneys were collected for analysis (Figure 1e and g). Immunofluorescence microscopy revealed clustering of Confetti$^+$ cells in the renal cortex of NTN mice and in the medulla of mice under HAD (Figure 1f and h). Each of these clusters expressed only 1 of the 4 Confetti colors, identifying them as clonally expanded cells.

Measurement of distribution of Confetti$^+$ MNPs

Using this setup, we analyzed the distribution and relationship of Confetti$^+$ MNPs by generating a rendering model on the maximum-intensity projection of the confocal stack for computational analysis (Figure 2a). Our algorithm measured the proximities of each individual Confetti$^+$ MNP to each same-color MNP and compared them with Monte Carlo–simulated data, where individual MNPs are assigned random colors, resulting in random distances (Figure 2a). We defined the recorded data that lay above the 98% of the Monte Carlo data points as a nonrandom distribution of same-color MNPs and considered them progeny of clonal proliferation[6] (Figure 2b). This procedure allowed inferring whether the distribution of adjacent same-color MNPs was due to clonal expansion or random chance.

Clonal proliferation of Confetti$^+$ MNPs in kidney disease

We next quantified clonal expansion of renal MNPs under disease conditions. First, we generated rendering models of renal cortex and medulla from healthy mice, NTN mice, and HAD mice (Supplementary Figure S3). The density of Confetti$^+$ MNPs in these models was subsequently calculated using the above-mentioned algorithm. In mice with NTN, we observed around 4 division events, meaning 4 same-color Confetti$^+$ MNPs derived from the same parent cell, in the cortex (Figure 2c and d), but not in the medulla (Figure 2e and f), consistent with the cortical location of inflamed glomeruli in glomerulonephritis. By contrast, mice under HAD showed MNP division events exclusively in the medulla (Figure 2c–f), where osmolarity is higher and adenine crystals are more likely to precipitate than in the cortex. No division event was seen in healthy mice (Figure 2c–f).

A total of 0.5% to 1% of blood monocytes were Confetti$^+$ at 1 week after tamoxifen injection (Supplementary Figure S4), consistent with previous reports in central nervous system disease models.[5,6] This was much lower than the 25% to 30% of renal MNPs carrying the label (Supplementary Figure S4), but we nevertheless wanted to verify that Confetti$^+$ MNPs were derived from tissue-resident macrophages. To this end, we treated Microfetti mice with tamoxifen and induced kidney disease after 8 weeks. At this late time point, hardly any monocytes retained the label, whereas still >20% of renal MNPs did so (Supplementary Figure S4). By utilizing the above-mentioned algorithm, the densities of Confetti$^+$ MNPs and corresponding Monte Carlo–simulated data were determined and subsequently used for calculation of the division event (Supplementary Figure S5). Under these conditions, the number of Confetti$^+$ MNP division events in the cortex of mice with NTN was about 3; and in the medulla of mice under an HAD, it was about 2.5 (Figure 2g–i). However, some Confetti$^+$ clones were also seen in healthy controls, presumably due to homeostatic proliferation of tissue-resident MNPs, as described.[9] Nevertheless, this verified that many Confetti$^+$ clones in both disease models were derived from tissue-resident MNPs.

Characterization of intrarenal Confetti$^+$ MNP clones and validation of proliferation

To determine whether the Confetti$^+$ clones were of the M1 or M2 differentiation type, we stained tissue sections for CD86 and CD206 (Supplementary Figure S6), respectively. We found that ≈25% of the Confetti$^+$ clones in NTN contained most cells expressing one of these markers (Figure 2j). In HAD mice, there were more CD86$^+$ M1 compared with CD206$^+$ M2 Confetti clones (Figure 2j). For further phenotypic characterization, we also ascertained that Confetti$^+$ clones in both, NTN and HAD, were F4/80$^+$ (Figure 1), CD11b$^+$, major histocompatibility complex II$^+$, and CD11c$^+$ (Supplementary Figure S6), consistent with tissue-resident MNPs.

Finally, we wanted to validate the local proliferation of MNP clones. To this end, we labeled proliferating cells by injecting

Figure 2 | (continued) gray, giving as boxes the 2nd to 98th percentiles of density. The data density (purple line) above the top gray box shows the nonrandom occurrence of same-color Confetti$^+$ MNPs, indicating clonal proliferation. (**c–f**) MNP proliferation in renal cortex (**c**) and medulla (**e**) based on same-color Confetti$^+$ cell density of healthy (left) and nephrotoxic serum (NTS; middle) 1 week after disease induction and HAD (right) 10 days after HAD treatment. In (**d**) and (**f**), the Confetti$^+$ cell densities were divided by the 98th percentile of MC values to determine clonal MNP proliferation in the renal cortex (**d**) and medulla (**f**). (**g–i**) Same experiment as (**c–f**), except that disease was induced at 8 instead of 1 week after inducing the Confetti label by tamoxifen (TAM) injection (**g**). Mean values are presented for groups of 3 to 5 mice. (**j**) Proportion of intrarenal CD86$^+$ M1 and CD206$^+$ M2 among the Confetti$^+$ clones in NTN and HAD mice, determined by fluorescence microscopy. Data presented as mean ± SD, and statistical analysis was performed by using Student *t* test. *$P < 0.05$. (**k**) Proportion of bromodeoxyuridine (BrdU)$^+$ among individual color Confetti$^+$ cells in kidneys from NTN and HAD mice, determined by flow cytometry. Data presented as mean ± SD. (**l,m**) Representative maximum-intensity projection of the confocal stack from BrdU-treated NTN (**l**) and HAD (**m**) mouse models. Green fluorescent protein (GFP) is in green, and BrdU is in magenta. Magnified individual channels of the rectangle region in the main panel are shown in the right panel. Bar = 50 μm (main panel) and 20 μm (inset panel). CFP, cyan fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; RFP, red fluorescent protein; YFP, yellow fluorescent protein. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

bromodeoxyuridine (BrdU) 5 and 6 days after NTN or 8 and 9 days after HAD induction and analyzed their kidneys for BrdU$^+$ cells. Flow cytometric analysis revealed that ≈35% of the Confetti$^+$ MNPs from NTN mice and 20% from HAD mice were BrdU$^+$ (Figure 2k; Supplementary Figure S7). To detect proliferation in clones expressing the Confetti labels, we performed immunofluorescence microscopy using an anti-GFP antibody that detects GFP and yellow fluorescent protein, because the BrdU staining protocol for tissue sections destroys the Confetti label. Indeed, we detected Confetti$^+$ clones containing BrdU$^+$ cells (Figure 2l and m). Moreover, we confirmed the presence of BrdU$^+$ cells also among clones of F4/80$^+$ MNPs in kidney sections (Supplementary Figure S8), further supporting local MNP proliferation. Finally, we also observed a higher proportion of CD86$^+$ M1 compared with CD206$^+$ M2 cells in BrdU$^+$ Confetti$^+$ cells (Supplementary Figure S9), consistent with the histologic result.

DISCUSSION

We here demonstrate that MNPs clonally expand in inflamed renal compartments during experimental glomerulonephritis and tubulointerstitial nephritis. Previous immunologic studies reported that tissue-resident MNPs can maintain their abundance by slow clonal proliferation in several uninflamed tissues of adult mice, including spleen, lung, and kidney, and in some studies, with a lower turnover rate, also microglia, Kupffer cells, and Langerhans cells.[2,5,6,9] The speed of this steady-state turnover was in the order of 8, 12, or 16 weeks or longer, depending on the labeling system used. This may explain why we failed to detect clonal expansion of renal MNPs in healthy mice after only 14 to 17 days.

A previous study had used Microfetti mice to show that microglia proliferated within 1 week after brain injury after unilateral facial nerve axotomy, compared with only once within 8 weeks under homeostatic conditions.[6] This suggested that tissue MNPs might clonally proliferate under inflammatory conditions also in other organs. Indeed, we here found that also kidney MNPs clonally expanded *in situ* after 1 week. These renal MNPs were mostly F4/80$^+$ CD11b$^+$ CD11c$^+$ major histocompatibility complex II$^+$, consistent with tissue-resident MNPs, previously classified as both dendritic cells and macrophages.[1] More importantly, 8 weeks after labeling, when hardly any Confetti$^+$ monocytes remained, almost as many intrarenal Confetti$^+$ clones were detected in both disease models. Some of these clones presumably had resulted from homeostatic proliferation,[9] implying that some Confetti$^+$ clones 1 week after labeling had originated from recruited monocytes. Nevertheless, these findings verified that many of the locally proliferating MNP clones had originated from tissue-resident cells.

Our findings show that the accumulation of disease-driving MNPs during nephritis partially results from clonal *in situ* proliferation at sites of inflammation. Although the NTN and HAD models are imperfect mimics of human crescentic glomerulonephritis or acute interstitial nephritis, these findings suggest that therapeutic strategies to treat nephritis solely by preventing the recruitment of circulating monocytes may be of limited effectiveness, because MNPs may still accumulate through local proliferation.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We acknowledge support by the Microscopy and the Flow Cytometry Core Facilities and the central animal facilities of the Medical Faculty of Bonn University. We thank our technician Melanie Eichler for the excellent technical support. This work was supported by the German Research Foundation (DFG) through SFB1192 number 264599542, IRTG2168 number 272482170, TRR237 number 369799452, FOR5427 number 466687329, KFO329 number 386793560, SFB1454 number 432325352, EXC2151 number 390873048, by a Gottfried Wilhelm Leibniz Award to CK, and by the Open Access Publication Fund of the University of Bonn.

AUTHOR CONTRIBUTIONS

JY and CK conceptualized the study and wrote the article. JY performed the experiments and the bioinformatic analysis. JL helped with the bioinformatic analysis. MP, UP, ZA, QM, and SvV provided essential tools and know-how. All authors discussed and interpreted the data.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Supplementary Figure S1. Multichannel micrograph of Confetti$^+$ mononuclear phagocytes (MNPs) in the kidney of Microfetti mice.

Supplementary Figure S2. Location of cellular compartments of Confetti-labeled mononuclear phagocytes (MNPs).

Supplementary Figure S3. Rendering model of confocal stack image of kidney from Microfetti mice.

Supplementary Figure S4. Flow cytometry analysis of Confetti labeling in Ly6c$^+$ blood monocytes and F4/80$^+$ renal mononuclear phagocytes (MNPs).

Supplementary Figure S5. Rendering model (top row) of confocal stack image of kidney from healthy and nephrotoxic nephritis (NTN) mice.

Supplementary Figure S6. Confocal images showing the expression of surface markers on Confetti-labeled mononuclear phagocytes (MNPs).

Supplementary Figure S7. Gating strategy of bromodeoxyuridine (BrdU)$^+$ cells (Figure 2k) among Confetti$^+$ cells in kidneys of Microfetti mice.

Supplementary Figure S8. Confocal images of bromodeoxyuridine (BrdU)$^+$ F4/80$^+$ mononuclear phagocytes (MNPs) in kidneys from nephrotoxic nephritis (NTN)– and high adenine diet (HAD)–treated Microfetti mice.

Supplementary Figure S9. Cell proportion of bromodeoxyuridine (BrdU)$^+$ CD86$^+$ M1 and CD206$^+$ M2 cells within Confetti$^+$ BrdU$^+$ cells from nephrotoxic nephritis (NTN)– and high adenine diet (HAD)–treated Microfetti mice.

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