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Time restricted feeding decreases renal innate immune cells and blood pressure in hypertensive mice

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Background: Renal innate immune cell accumulation and inflammation are associated with hypertension. Time restricted feeding (TRF) has been reported to decrease inflammation and blood pressure. Whether TRF can decrease blood pressure by decreasing renal innate immune cells in hypertension is unknown.

Methods and results: We determined whether TRF can decrease blood pressure in two separate mouse models of hypertension, N(G)-nitro-L-arginine methyl ester hydrochloride-induced hypertension (LHTN) and salt-sensitive hypertension (SSHTN). Once hypertension was established after 2 days, TRF (12-h food/12-h no food) for 4 weeks significantly decreased systolic blood pressure in both LHTN and SSHTN mice despite no differences in the amount of food eaten or body weight between groups. Activated macrophages and dendritic cells in the kidneys of both LHTN and SSHTN mice were decreased significantly in mice that underwent TRF. This was associated with an improvement in kidney function (decreased serum creatinine, decreased fractional excretion of sodium, and increased creatinine clearance) which achieved significance in LHTN mice and trended towards improvement in SSHTN mice.

Conclusions: Our findings demonstrate that TRF can significantly decrease renal innate immune cells and blood pressure in two mouse models of hypertension.

Keywords: blood pressure, dendritic cells, kidney function, macrophages, time restricted feeding

Abbreviations: LHTN, L-NAME-induced hypertension; PKD, polycystic kidney disease; SBP, systolic blood pressure; SSHTN, salt-sensitive hypertension; T2D, type 2 diabetes mellitus; TRF, time restricted feeding

INTRODUCTION

Hypertension, a condition where blood pressure is consistently higher than 130/80 mmHg, affects almost half of the adult population worldwide. It is currently the largest contributing factor to cardiovascular deaths in the world and can lead to many risks such as heart disease and kidney dysfunction if left untreated. Disparities in hypertension are constantly increasing and vary drastically among high-, middle-, and low-income countries worldwide [1]. In higher income countries, awareness and control of hypertension have been seen to increase significantly, whereas low- and middle-income countries have seen a small decrease [1]. In areas where hypertension is most prevalent, the development of cost-effective treatments is critical.

Numerous factors contribute to the development of hypertension, but activation of the innate immune system and renal infiltration of pro-inflammatory innate immune cells, such as macrophages and dendritic cells, have been identified in experimental models and patients with hypertension [2,3]. Activated immune cells infiltrate the kidney, pro-inflammatory cytokines affect renal sodium handling, contribute to kidney dysfunction, and propagate the hypertension [4]. Medications that suppress the immune system have been reported to lower blood pressure in animals and humans. Also, the depletion of innate immune cells in hypertensive rodents either genetically or pharmacologically also decreases blood pressure [5–7]. However, expensive therapeutics that cause global immunosuppression are not ideal to treat hypertension throughout the world, thus the need for an easy and safe therapeutic strategy remains.

Time restricted feeding (TRF), or 'time restricted eating' in humans, is a subset of intermittent fasting that restricts the consumption of food to a specific window of time each day without restricting the timing of water intake, thereby incorporating a regular fasting period into each day [8]. TRF has been reported to be beneficial in the management of specific chronic conditions, especially metabolic diseases [9]. Types of intermittent fasting have been reported to

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reduce various cardiometabolic endpoints in both rodents and humans throughout different studies [8]. Similar health benefits, such as improvement of insulin sensitivity, a reduction in inflammation markers, and reduced cholesterol and triglycerides have been conveyed throughout intermittent fasting studies in humans [10–12]. Recent research demonstrates that TRF plays a role in managing cardiovascular disease by lowering blood pressure in humans, but the mechanisms by which this occurs have not been clearly elucidated [10,12]. Due to the benefits of TRF on the cardiovascular system, it is possible that TRF could be included as an easy and no-cost intervention in the management of chronic hypertension.

In this study, we investigated whether TRF can alter renal innate immune cells and blood pressure in two independent mouse models of hypertension. We hypothesized that TRF would decrease renal innate immune cells and lower blood pressure in mice with the nitric oxide synthase inhibitor L-arginine methyl ester hydrochloride (L-NAME)-induced hypertension (LHTN) or salt-sensitive hypertension (SSHTN).

METHODS

The data that support the findings of this study are available from the corresponding author (B.M.M.) upon request.

Animal care

All animal use protocols were approved by the Texas A&M University IACUC and were performed in accordance with the NIH Guide for the Care and Use and Care of Laboratory Animals.

Mice

C57BL/6J mice were purchased from Jackson Labs at age 8–10 weeks and allowed to acclimate for 2 weeks. Male and female mice were equally and randomly assigned to the four experimental groups. There were four males and four females in each of the LHTN groups and there were three males and three females in each of the SSHTN groups. For flow cytometry and serum and urine K^+ and Cl^- measures, there were three mice in each of the LHTN groups.

Experimental design

N(G)-Nitro-L-arginine methyl ester hydrochloride-induced hypertension

Male and female mice were made hypertensive by providing L-NAME (0.5 mg/ml; Sigma, St. Louis, Missouri, USA) in the drinking water. After 2 days of L-NAME treatment in which systolic blood pressure (SBP) is significantly increased, some mice remained on *ad libitum* access to food and water (control) while others had their food removed daily from 0800 until 2000 h (TRF) for 4 weeks. Weekly SBPs were determined using the tail-cuff method as described below. At the end of 4 weeks, mice were placed in diuresis cages and allowed to acclimate for 24 h and then urine was collected from individual mice over 24 h. Urine analysis was not performed if $<100\,\mu$l was collected.

Salt-sensitive hypertension

Male and female mice received L-NAME in the drinking water for 2 weeks, followed by a 2-week washout, and then a 4% salt diet for 4 weeks. After two days of beginning the 4% salt diet in which SBP is significantly increased, some mice remained on *ad libitum* access to 4% salt food and water (control) whereas others had their 4% salt food removed daily from 0800 until 2000 h (TRF) for 4 weeks. Weekly SBPs were determined by tail-cuff. At the end of 4 weeks, mice were placed in diuresis cages and allowed to acclimate for 24 h and then urine was collected from individual mice over 24 h. Again, urine analysis was not performed if $<100\,\mu$l was collected.

Food consumption determination

Food consumption was determined by weighing the food every morning at 0800 h. The amount of food remaining was subtracted from the night before and divided by the number of mice per cage to determine the average food consumed per mouse in grams per day. Final body weights were measured prior to euthanization.

Blood pressure determination

SBP was measured using a IITC Life Science noninvasive tail-cuff blood pressure system. Mice were acclimated in a quiet area where the procedures were performed. Mice were placed in prewarmed (34°C) restrainers and allowed to acclimate for 5 min prior to recordings. SBP readings were determined by two independent, blinded investigators.

Flow cytometry

Kidneys were harvested and minced following decapsulation. The minced kidneys were digested in buffer containing Collagenase D (Roche Sigma, St. Louis, Missouri, USA) and Dispase II (Sigma) at 37°C for 35 min with constant disruption from a gentleMACS Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, cells were filtered through 100 μm and 40 μm strainers to obtain single cell suspensions. Red blood cells were lysed in ammonium–chloride–potassium (ACK) lysing buffer (Life Technologies, Carlsbad, California, USA). Splenocytes were similarly isolated. Nonviable cells were stained with Ghost Dye Red 710 (Tonbo Biosciences, San Diego, California, USA) to allow for their exclusion in later analyses. Nonspecific Fc binding was blocked using an antimouse CD16/CD32 antibody (BD Pharmingen, San Jose, California, USA), following which cells were incubated with fluorescent-conjugated antibodies against CD45, CD11b, CD11c, F4/80, CD38, CD64, Gr1, and CD206 for 30 min on ice. All antibodies were purchased from either BD Pharmingen or BioLegend and the details are provided in Table S1, Supplemental Digital Content, http://links.lww.com/HJH/B970. Data were acquired on a BD LSR Fortessa X-20 flow cytometer using FACS DIVA software (BD Biosciences) and analyzed using Flow Jo v7.6.2 (FlowJo, LLC, Ashland, Oregon, USA). Results are expressed as a percentage of CD45+ cells per kidney or spleen. The gating strategy can be seen in Fig. S1, Supplemental Digital Content, http://links.lww.com/HJH/B970.

Urine and serum analysis

Serum and urine (24-h collection) samples were analyzed for creatinine by direct potentiometry using a P/ACE MDQ Plus Capillary Electrophoresis System (Sciex, Redwood City, California, USA) at the University of Texas-Southwestern George M. O'Brien Kidney Research Core Center. Samples were analyzed for Na^+, K^+, and Cl^- concentration by capillary electrophoresis using a DxC 700 AU Chemistry Analyzer (Beckman Coulter, Brea, California, USA) at Texas A&M University's Rodent Preclinical Phenotyping Core. Serum levels of interleukin (IL)-1b and IL-6 were determined by running samples through a ProcartaPlex Multiplex Immunoassay (Invitrogen, Waltham, Massachusetts, USA), following the manufacturer's recommended protocol. Results were acquired using a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, California, USA) and Bio-Plex Manager 5.0 software (Bio-Rad).

Statistical methods

Results are presented as dot plots with mean line and/or graphs displaying mean ± SEM. The two-tailed unpaired Student's *t* test was used for comparison of means between control and TRF for each form of hypertension. The criterion for significance was set at $P < 0.05$. All analyses were performed using GraphPad Prism 7 software (La Jolla, California, USA).

RESULTS

Some believe that the beneficial effects of TRF are from a reduction in food consumption while others have reported beneficial effects of TRF even with isocaloric diets. We measured the amount of food consumed per day per mouse and there were no significant differences in the LHTN or SSHTN groups between the mice that underwent TRF and those that had *ad libitum* access to food 24 h a day (Fig. 1a). As expected, the SSHTN groups tended to eat more than the LHTN groups. There were no differences in body weight (Fig. 1b) or 24-h urine volume (Fig. 1c) in LHTN or SSHTN mice after undergoing 4 weeks of TRF compared to *ad libitum* feeding. There were also no differences in spleen weight/body weight ratio (Fig. S2a, Supplemental Digital Content, http://links.lww.com/HJH/B970), total weight of the kidneys (Fig. S2b, Supplemental Digital Content, http://links.lww.com/HJH/B970), or heart weight (Fig. S2c, Supplemental Digital Content, http://links.lww.com/HJH/B970) in either LHTN or SSHTN groups undergoing TRF compared to LHTN or SSHTN groups that had *ad libitum* access to food. Lastly, there were no significant differences in serum (Fig. S3a, Supplemental Digital Content, http://links.lww.com/HJH/B970) or urine (Fig. S3b, Supplemental Digital Content, http://links.lww.com/HJH/B970) K^+ or in serum (Fig. S4a, Supplemental Digital Content, http://links.lww.com/HJH/B970) or urine (Fig. S4b, Supplemental Digital Content, http://links.lww.com/HJH/B970) Cl^- between groups.

TRF decreased SBP significantly in both LHTN (Fig. 2a) and SSHTN (Fig. 2b) mice. SBP in the LHTN + TRF group was decreased significantly by week 3 and remained blunted through week 4 (Fig. 2a and c). SBP in the SSHTN + TRF group was decreased after only 1 week and remained blunted through all 4 weeks (Fig. 2b and d). The increased speed with which TRF treatment decreased blood pressure in SSHTN mice could be attributed to the

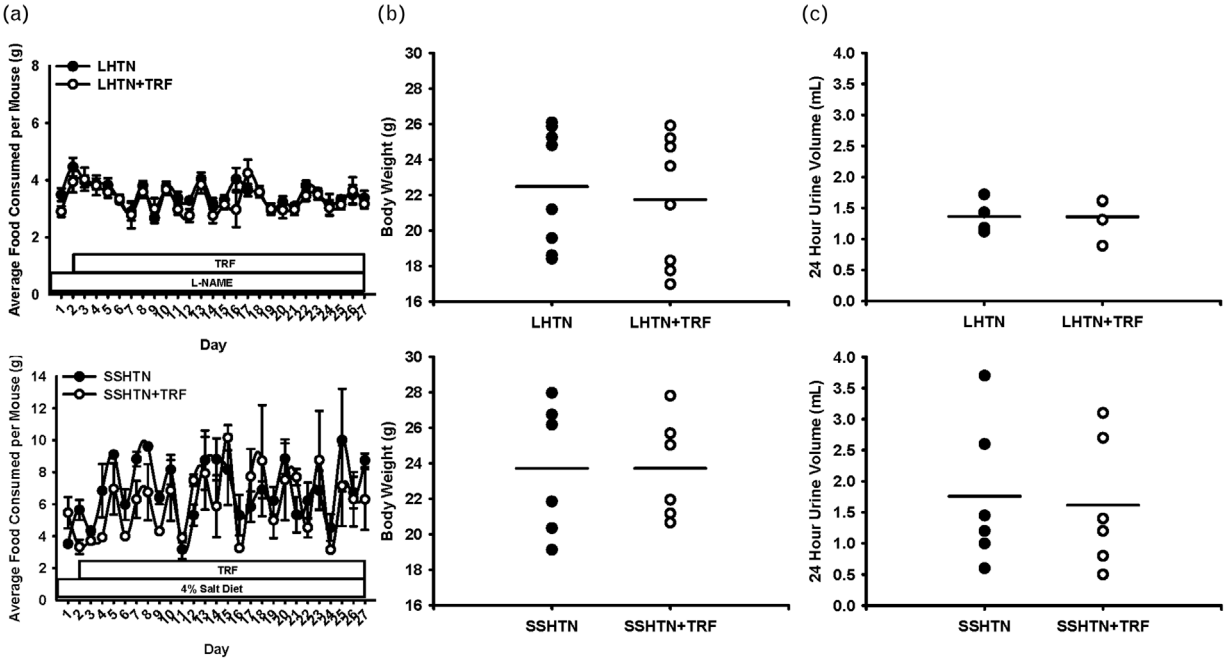

FIGURE 1 TRF did not alter food consumption, body weight, or urine volume. (a) Average food consumed per mouse per day, (b) body weight at the end of the 4 weeks, and (c) 24-h urine volume at the end of the 4 weeks in LHTN and SSHTN mice that either had *ad libitum* access to food and water or underwent TRF. Results are expressed as dot plots with mean line or mean ± SEM. $n = 8$ mice for each LHTN group and $n = 6$ mice for each SSHTN group. LHTN, L-NAME-induced hypertension; SSHTN, salt-sensitive hypertension; TRF, time restricted feeding; SEM, standard error of mean.

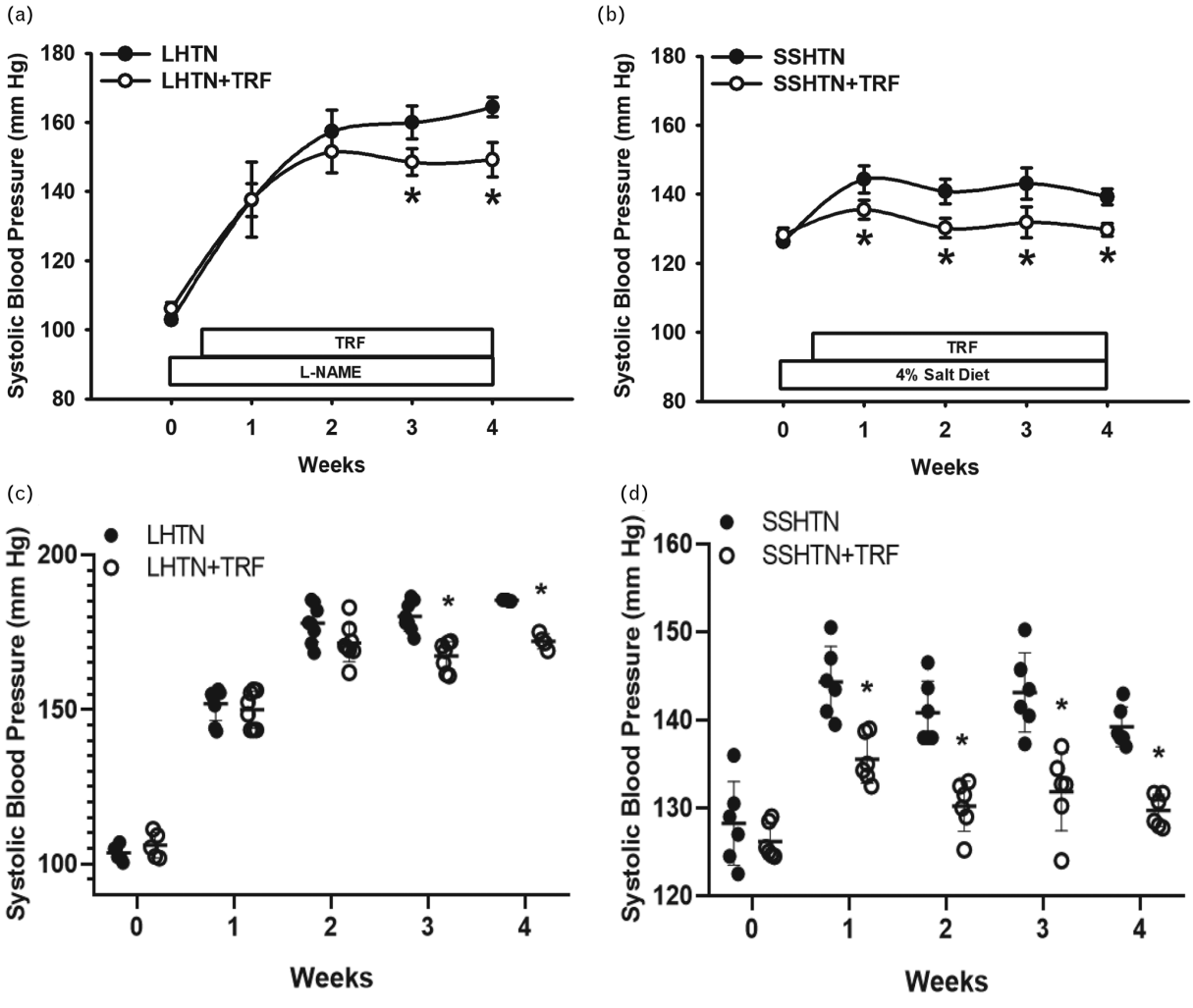

FIGURE 2 TRF significantly blunted blood pressure in hypertensive mice. Weekly systolic blood pressure measures in (a) LHTN and (b) SSHTN mice that either had *ad libitum* access to food and water or underwent TRF. Blood pressure readings are organized into dot plots representing mice from the (c) LHTN and (d) SSHTN groups. Results are expressed as dot plots with mean ± SEM. $n = 8$ mice for each LHTN group and $n = 6$ mice for each SSHTN group. * $P < 0.05$ compared to the non-TRF group. LHTN, L-NAME-induced hypertension; SSHTN, salt-sensitive hypertension; TRF, time restricted feeding; SEM, standard error of mean.

less aggressive nature of salt as a hypertensive stimulus when compared to L-NAME. L-NAME is known to induce severe hypertension quickly (in some cases, after only 1 day) and could have created more dysfunction for the TRF treatment to combat [13].

Renal innate immune cells that were decreased significantly by TRF in either LHTN or SSHTN mice are displayed in Fig. 3. Activated macrophages (CD45+CD11b+F4/80+CD38+) were decreased significantly in kidneys of SSHTN mice who underwent TRF (Fig. 3a). CD45+CD11b+F4/80+CD64+ macrophages were decreased significantly in kidneys of LHTN mice who underwent TRF (Fig. 3b). Dendritic cells (CD45+F4/80-CD11c+) were decreased significantly in kidneys of SSHTN mice who underwent TRF (Fig. 3c). Similarly, activated dendritic cells (CD45+F4/80-CD11c+CD38+) were decreased significantly in kidneys of LHTN mice who underwent TRF (Fig. 3d). Lastly, the innate immune cell populations of CD45+CD11b^{low}CD11c- macrophages (Fig. 3e) and CD45+CD11c+ dendritic cells (Fig. 3f) were decreased significantly in the kidneys of LHTN mice who underwent TRF. Renal innate immune cells that were not significantly decreased by TRF in either LHTN or SSHTN are displayed in Fig. S5, Supplemental Digital Content, http://links.lww.com/HJH/B970 and Fig. S6, Supplemental Digital Content, http://links.lww.com/HJH/B970. These included CD45+ immune cells (Fig. S5a, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+Gr1+ myeloid-derived suppressor cells (Fig. S5b, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD64+Gr1+ monocytes (Fig. S5c, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD64+Gr1+CD38+ activated monocytes (Fig. S5d, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b^{high}CD11c- macrophages (Fig. S5e, Supplemental Digital Content, http://links.lww.com/HJH/B970), which were increased significantly in kidneys of SSHTN mice, CD45+CD11b+F4/80+ macrophages

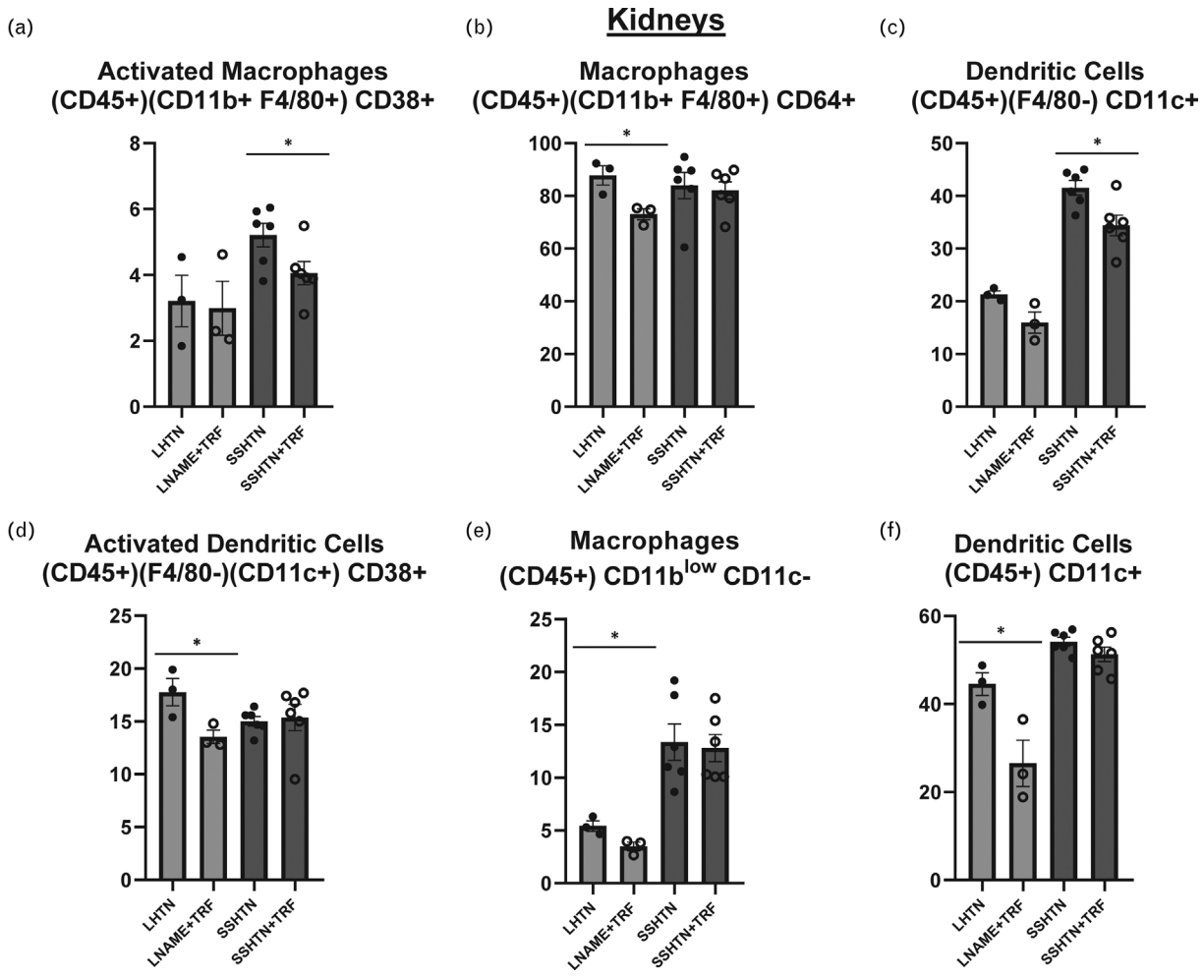

FIGURE 3 Renal innate immune cells that were significantly altered by TRF. (a) CD45+CD11b+F4/80+CD38+, (b) CD45+CD11b+F4/80+CD64+, (c) CD45+F4/80-CD11c+, (d) CD45+F4/80-CD11c+CD38+, (e) CD45+CD11b^{low}CD11c-, and (f) CD45+CD11c+. Results are expressed as dot plots with mean ± SEM. $n = 3$ mice for each LHTN group and $n = 6$ mice for each SSHTN group. TRF, time restricted feeding; SEM, standard error of mean.

(Fig. S5f, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD206-CD11c+ M1 macrophages (Fig. S5g, Supplemental Digital Content, http://links.lww.com/HJH/B970), and CD45+CD11b+F4/80+CD206+CD11c-M2 macrophages (Fig. S5h, Supplemental Digital Content, http://links.lww.com/HJH/B970). Additional renal innate immune cells that were not changed by TRF in either LHTN or SSHTN include CD45+F4/80− immune cells (Fig. S6a, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+F4/80+ macrophages (Supplemental Digital Content, Fig. S6b, http://links.lww.com/HJH/B970), CD45+CD11b+ myeloid cells (Fig. S6c, Supplemental Digital Content, http://links.lww.com/HJH/B970), and CD45+CD11b+CD11c+ myeloid dendritic cells (Fig. S6d, Supplemental Digital Content, http://links.lww.com/HJH/B970).

Splenic innate immune cells were also altered significantly by TRF, and these are displayed in Fig. S7, Supplemental Digital Content, http://links.lww.com/HJH/B970. M2 macrophages (CD45+CD11b+F4/80+CD206+CD11c−) were decreased in spleens of SSHTN mice who underwent TRF (Fig. S7a, Supplemental Digital Content, http://links.lww.com/HJH/B970). There were four cell populations that were increased significantly in the spleens of SSHTN mice who underwent TRF: CD45+CD11b+F4/80+ macrophages (Fig. S7b, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+F4/80-CD11c+ dendritic cells (Fig. S7c, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+ myeloid cells (Fig. S7d, Supplemental Digital Content, http://links.lww.com/HJH/B970), and CD45+CD11b+CD11c+ myeloid dendritic cells (Fig. S7e, Supplemental Digital Content, http://links.lww.com/HJH/B970). Splenic innate immune cells that were unchanged by TRF in either LHTN or SSHTN are displayed in Fig. S8, Supplemental Digital Content, http://links.lww.com/HJH/B970 and Fig. S9, Supplemental Digital Content, http://links.lww.com/HJH/B970. These included CD45+ immune cells (Fig. S8a, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD38+ activated macrophages (Fig. S8b, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD64+Gr1+ monocytes (Fig. S8c, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+CD64+Gr1+CD38+ activated monocytes (Fig. S8d, Supplemental Digital Content,

http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+ Gr1+ myeloid-derived suppressor cells (Fig. S8e, Supplemental Digital Content, http://links.lww.com/HJH/B970), and CD45+CD11b+F4/80-CD206-CD11c+ M1 macrophages (Fig. S8f, Supplemental Digital Content, http://links.lww.com/HJH/B970). Additionally, CD45+F4/80- immune cells (Fig. S9a, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+F4/80-CD11c+CD38+ activated dendritic cells (Fig. S9b, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+F4/80+ macrophages (Fig. S9c, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b+F4/80+ CD64+ macrophages (Fig. S9d, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b^{high} CD11c- macrophages (Fig. S9e, Supplemental Digital Content, http://links.lww.com/HJH/B970), CD45+CD11b^{low} CD11c- macrophages (Fig. S9f, Supplemental Digital Content, http://links.lww.com/HJH/B970), and CD45+ CD11c+ dendritic cells (Fig. S9g, Supplemental Digital Content, http://links.lww.com/HJH/B970) were also unchanged by TRF in either LHTN or SSHTN mice.

To investigate whether these innate immune cell changes were associated with a reduction in inflammation, we measured serum cytokine levels. Serum from mice that underwent TRF in both hypertensive groups had significantly decreased levels of the pro-inflammatory cytokine IL-6 (Fig. 4a). Additionally, SSHTN mice who underwent TRF had decreased serum levels of the pro-inflammatory cytokine IL-1b (Fig. 4b). Decreased circulating pro-inflammatory cytokines support the known finding that TRF decreases inflammation, which may have contributed in part to the significant decrease in blood pressure.

Next, we determined whether these reductions in renal innate immune cells and pro-inflammatory cytokines were associated with improved kidney function. TRF significantly decreased serum creatinine in LHTN mice and tended to decrease serum creatinine in SSHTN mice, however it did not reach statistical significance (Fig. 5a). TRF also significantly decreased fractional excretion of Na^+ (FENa) in LHTN mice and again, tended to decrease FENa in SSHTN mice but this did not reach statistical significance (Fig. 5b). Lastly, TRF significantly increased creatinine clearance (CrCl), an estimate of glomerular filtration rate, in LHTN mice and tended to have the same effect in SSHTN mice yet this did not reach statistical significance (Fig. 5c). These results demonstrate that kidney function was improved by TRF.

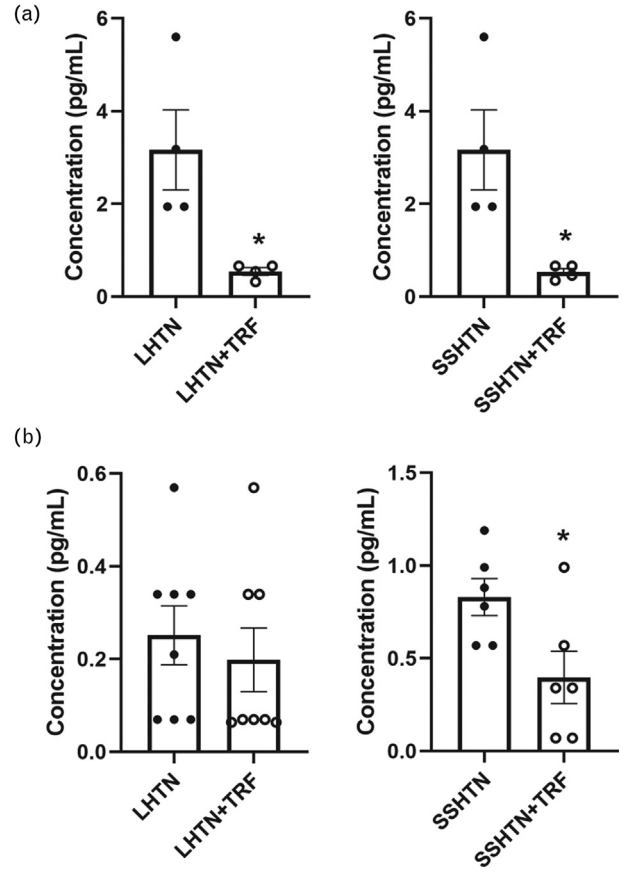

FIGURE 4 TRF decreased circulating pro-inflammatory cytokines in hypertensive mice. Serum levels of pro-inflammatory cytokines (a) IL-6 and (b) IL-1b in mice with LHTN and SSHTN. Results are expressed as dot plots with mean ± SEM. For IL-6 calculations, $n = 4$ for each group. For IL-1b calculations, $n = 8$ for each LHTN group and $n = 6$ for each SSHTN group. * $P < 0.05$ compared to the non-TRF group. LHTN, L-NAME-induced hypertension; SSHTN, salt-sensitive hypertension; TRF, time restricted feeding; SEM, standard error of mean.

DISCUSSION

The major findings of the current study are that: TRF did not alter the total amount of food consumed or body weight but was able to significantly decrease blood pressure in two independent mouse models of hypertension, the TRF-induced blunting of hypertension in mice was associated with decreased renal innate immune cells, and TRF tended to improve kidney function.

Many studies have determined the effects of TRF on cardiometabolic health. Most studies report that TRF significantly reduces blood pressure in various conditions. A study examined a 16-h TRF protocol in 23 healthy college-aged men for 4 weeks [14]. After TRF intervention, McAllister and colleagues reported a significant blunting of systolic blood pressure (119 ± 11 mmHg to 114 ± 10 mmHg) along with improvement in other health markers [14]. Not only was it shown that TRF could reduce blood pressure in healthy individuals, Wilkinson and colleagues also reported the same trend when TRF was completed in patients with metabolic syndrome [15]. After a 12 week, 10-h TRF was completed on 19 metabolic syndrome patients, they reported a significant decrease in both systolic (−5.12 ± 9.51 mmHg [−4%]) and diastolic (−6.47 ± 7.94 mmHg [−8%] blood pressure [15]. Additionally, Sutton and colleagues performed an early TRF experiment where patients with prediabetes attempted an early TRF schedule [10]. Participants ate before 1500 h in a 6-h daily eating period for 5 weeks. Following a 5-week, 12-h control TRF, they reported similar significant reductions in systolic and diastolic blood pressure [10]. Lastly, metabolic risk factors as well as blood pressure were explored in obese adults during an 8-h TRF intervention for 12 weeks [16]. Gabel and colleagues reported a significant reduction in systolic blood pressure (−7 ± 2 mmHg) after intervention [16].

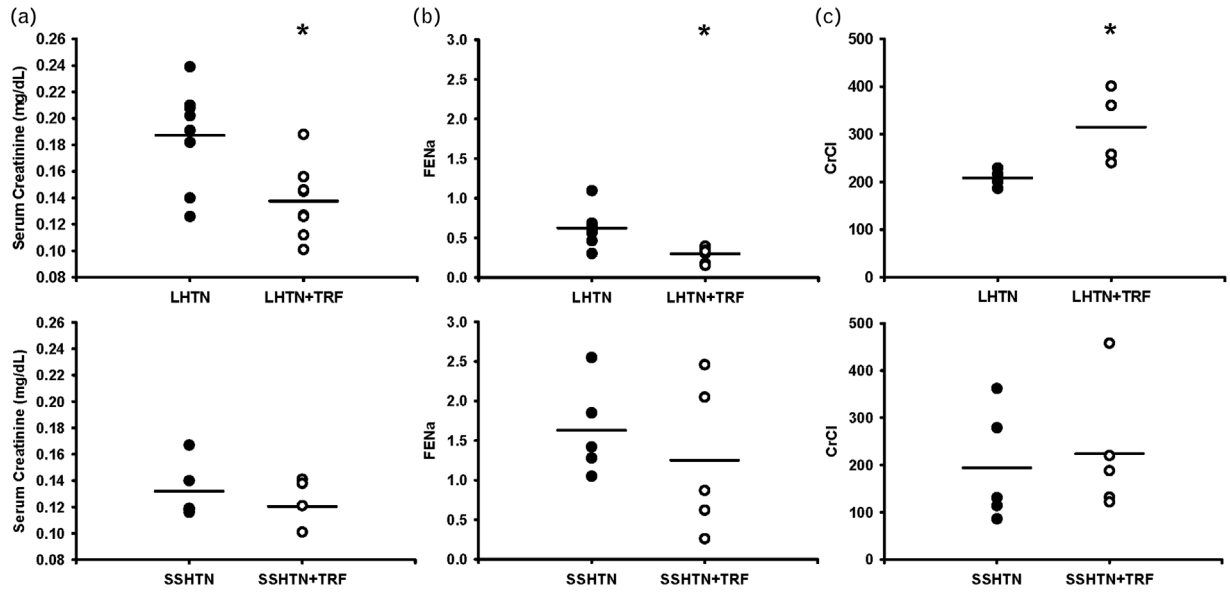

FIGURE 5 TRF improved renal sodium handling in hypertensive mice. (a) Serum creatinine, (b) fractional excretion of sodium (FENa), and (c) creatinine clearance (CrCl) at the end of the 4 weeks in LHTN and SSHTN mice that either had *ad libitum* access to food and water or underwent TRF. Results are expressed as dot plots with mean line. $n = 8$ mice for each LHTN group and $n = 6$ mice for each SSHTN group. * $P < 0.05$ compared to the non-TRF group. LHTN, L-NAME-induced hypertension; SSHTN, salt-sensitive hypertension; TRF, time restricted feeding; SEM, standard error of mean.

Despite the numerous studies that reported a significant decrease in blood pressure, there is one that did not report any significant change after TRF intervention. Cienfuegos and colleagues performed an 8-week, 4- and 6-h TRF protocol in adults with obesity where no significant difference in systolic or diastolic blood pressure was reported [17]. However, an 18–20 h fast may be too stressful and severe of a restriction for humans, resulting in the opposite trend that we and others observed. Nonetheless, the results of our study and others suggest that TRF seems to effectively reduce blood pressure in a variety of people with different health backgrounds.

Many studies have explored the benefits of TRF. Some say that the benefits of TRF are due a reduction in the amount of food consumed, but others have shown improvements in cardiometabolic markers and protection from pathological conditions, even with the use of isocaloric diets [18,19]. In our study, comparable body weights among the mice indicate that there was no significant difference in the amount of food consumed per mouse per day between the control and TRF groups. However, it must be noted that the only way to definitively track food consumption by individual mice would be to house them individually. This experimental design was not compatible with our study, as individually housed mice demonstrate psychological and physiological differences when compared to group-housed mice. We attempted to combat this limitation by allowing unlimited access to food (aside from the 12-h fasting window observed by the TRF mice). When food was in the cages, mice could consume as much as they pleased. Group-housed male mice have established dominance hierarchies which could impact the amount of food that subdominant animals were able to access. However, it is highly unlikely that social dynamics impacted consumption to the point of affecting data, as indicated by comparable body weights at the conclusion of the study and the fact that the mice were littermates who had grown up together. Our study demonstrates that the beneficial effects of TRF were not due to a reduction in food consumption; thus, we explored other mechanisms.

Renal infiltration of innate immune cells plays a critical role in the pathogenesis of hypertension. The pro-inflammatory cytokines produced by these immune cells and certain intrinsic renal cells exacerbate dysfunction by several mechanisms, including altering sodium handling. Cytokines can directly interact with sodium transporters, leading to upregulation of transporter expression and activity which results in increased sodium reabsorption and higher blood pressure [20]. These cytokine-transporter interactions may also be indirect in nature. Zhang *et al.* reported that by preventing the maturation of intrarenal myeloid cells into nitric oxide-producing macrophages, IL-1 receptor activation decreases nitric oxide production and enhances sodium reabsorption via NKCC2 [21]. IL-6 can increase sodium reabsorption both directly through interactions with the epithelial sodium channel and indirectly through activation of the renin-angiotensin system [22,23]. Pro-inflammatory cytokines contribute to hypertension and increased sodium reabsorption, but mechanisms differ for each cytokine and are currently under investigation. TRF has been reported to decrease cytokine levels in many different disease conditions, and in this study we observed that TRF decreased serum IL-1b and IL-6 levels in hypertensive mice [24–26]. Considering what is known about cytokine participation in renal sodium handling, decreased cytokine levels would lead to less retained sodium and decreased blood pressure. This mechanism can be seen at work in the current study.

Studies have reported that TRF is associated with a decrease in inflammation [27]. However, most studies measured circulating cytokines as markers and mediators of a

general state of inflammation. Few studies have examined immunity and immune cell changes, and none to our knowledge have examined renal innate immune cell changes resulting from TRF. Recently, TRF was reported to induce immune cell autophagy, or the 'self-eating' of old and ineffective immune cells, during the fasting portion. This removal resulted in stem cell activation and the 'refreshing' of the immune system [28]. A recent study similar to ours reported that mice that underwent 12-h TRF for 4 weeks had a significantly increased bacteria killing capacity, which is primarily mediated by the innate immune system [29]. Studies that have examined the effects of TRF on innate immune cells are very limited. In young and aged men, 12-h fasting significantly decreased both CD16+ and CD56+ NK cells [11]. With respect to monocytes, macrophages, and dendritic cells, Jordan and colleagues reported that fasting in both humans and mice significantly decreased circulating monocytes and dendritic cells [30]. Additionally, they reported that fasting for as little as 4 h in mice significantly decreased macrophages in various tissues, however the kidneys were not examined. Lastly, Lee *et al.* performed a study in which mice were fed a high fat diet for 8 weeks and some mice underwent a 14-h TRF [31]. They reported that TRF significantly decreased adipose tissue macrophages, which were CD45+CD11b+F4/80+, as well as CD11c+ macrophages, similar to our current findings of these same cells being decreased significantly in the kidneys of hypertensive mice that underwent a 12-h TRF for 4 weeks. Taken together, TRF appears to reduce innate immune cells, and this may contribute to the decrease in inflammation and blood pressure.

To our knowledge, there are no previous studies that specifically examined renal function following TRF in the context of hypertension. However, the effects of renal function on TRF have been studied in other conditions such as polycystic kidney disease (PDK) and diabetes. PDK is a genetic condition that promotes the development of renal cysts, which ultimately decreases renal function over time. Studies investigating the effects of TRF have found that this intervention has a beneficial impact on the progression of PDK. One study discovered that TRF inhibits the development of renal cysts by correcting signaling pathways that are negatively affected by PDK, specifically mTOR and STAT signaling, which improves kidney function [32]. Another study suggests the improvement to signaling pathways such as mTOR are due to regular TRF-induced ketosis, which is more easily inducible in healthy renal cells than in pathogenic cystic cells [33]. These studies suggest that TRF has a beneficial effect on PDK by relying upon the healthy metabolic homeostatic mechanisms that are present in healthy renal cells but are absent in pathogenic cystic cells, which promotes healthy cell signaling pathways which improve kidney function.

Diabetes, specifically Type 2 Diabetes Mellitus (T2D), is the leading cause of chronic kidney disease due to the damage that poorly controlled T2D causes to renal blood vessels, which ultimately contributes to hypertension, decreased GFR, and even end-stage renal disease [34,35]. The effects of utilizing TRF to prevent and treat diabetes is well documented. TRF has been reported to impact circulating blood glucose levels by decreasing both mean fasting glucose and HbA1c levels [15,36]. Furthermore, TRF increases β cell responsiveness to blood glucose levels, which decreases insulin resistance [10]. These findings suggest that TRF has a role in the protection and treatment against T2D, which ultimately reduces the negative impacts that T2D has on kidney function.

Perspectives

TRF for 4 weeks was sufficient to significantly decrease renal innate immune cells and blood pressure. TRF may serve as a safe, no-cost, and effective way to lower blood pressure where medications are not readily accessible.

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Conflicts of interest

There are no conflicts of interest.

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