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## NOX2-derived Reactive Oxygen Species in Immune Cells Exacerbates Salt-sensitive Hypertension

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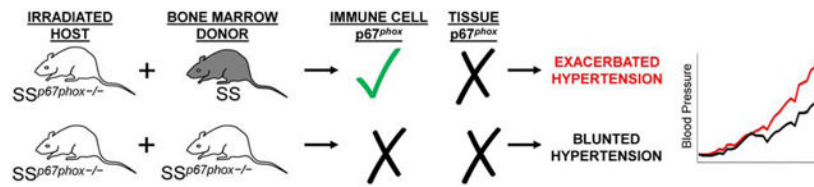
### Abstract

Previous studies utilizing the  $SS^{p67phox-/-}$  rat have demonstrated the importance of systemic NADPH oxidase NOX2-derived reactive oxygen species (ROS) production in the pathogenesis of Dahl Salt-Sensitive (SS) hypertension and renal damage. It is established that the immune system contributes to the development of SS hypertension and our laboratory has observed an enrichment of NOX2 subunits in infiltrating T cells. However, the contribution of immune cell-derived ROS in SS hypertension remains unknown. To assess the role of ROS in immune cells,  $SS^{p67phox-/-}$  rats underwent total body irradiation and received bone marrow transfer from either SS (+SS) or  $SS^{p67phox-/-}$  (+ $SS^{p67phox-/-}$ ) donor rats. Demonstrated in a respiratory burst assay, response to phorbol 12-myristate 13-acetate stimulus (135  $\mu$ M) was 10.2-fold greater in peritoneal macrophages isolated from +SS rats compared to nonresponsive + $SS^{p67phox-/-}$  cells, validating that +SS rats were capable of producing NOX2-derived ROS in cells of hematopoietic origin. After 3 weeks of high salt challenge, there was an exacerbated increase in mean arterial pressure in +SS rats compared to + $SS^{p67phox-/-}$  control rats (176.1 $\pm$ 4.7 vs 147.9 $\pm$ 8.4 mmHg, respectively), which was accompanied by a significant increase in albuminuria (168.3 $\pm$ 23.7 vs 107.0 $\pm$ 20.4 mg/day) and renal medullary protein cast formation (33.2 $\pm$ 4.7 vs 8.1 $\pm$ 3.5%). Interestingly, upon analysis of renal immune cells, there was trending increase of CD11b/c+ monocytes and macrophages in the kidney of +SS rats (4.7 $\pm$ 0.4 vs 3.5 $\pm$ 0.5  $\times 10^6$  cells/kidney, +SS vs + $SS^{p67phox-/-}$ ,  $p=0.06$ ). These data altogether demonstrate that immune cell production of NOX2-derived ROS is sufficient to exacerbate Dahl SS hypertension, renal damage, and renal inflammation.

### Graphical Abstract

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## Keywords

Hypertension; reactive oxygen species; immune cells; renal damage; NADPH oxidase; p67<sup>phox</sup>

## Introduction

The inappropriate production of reactive oxygen species (ROS) is known to contribute to the development of many pathologies, especially cardiovascular diseases [1], and has been implicated in the progression of hypertension and renal damage in the Dahl salt-sensitive (SS) rat. Specifically, increased production of superoxide and hydrogen peroxide measured via microdialysis has been demonstrated in the renal medulla of SS rats [2, 3]. A major source of ROS comes from the NOX2 isoform of NADPH oxidase, a multi-subunit enzyme comprised of membrane (gp91<sup>phox</sup> and p22<sup>phox</sup>) and cytosolic subunits (p47<sup>phox</sup>, p67<sup>phox</sup>, p40<sup>phox</sup>, and Rac 1 or 2) [4]. *p67<sup>phox</sup>* was identified as a candidate gene in studies involving congenic Dahl SS rats generated through the substitution of chromosomes from salt-resistant Brown Norway rats into the background of disease-prone SS rats [5, 6]. Furthermore, SS rats have been shown to exhibit higher NADPH oxidase activity in the outer medulla compared to salt-resistant control rats [7] and have increased expression of the p67<sup>phox</sup> subunit of the enzyme [8]. Null mutation of the *p67<sup>phox</sup>* gene in the Dahl SS (SS *p67<sup>phox</sup>*<sup>-/-</sup>) rat by zinc finger nuclease technology resulted in a reduction in salt-induced mean arterial pressure and renal injury compared to SS *p67<sup>phox</sup>*<sup>+/+</sup>, providing the first evidence that ROS production specifically from the NOX2 isoform of NADPH oxidase is crucial for the development of salt-sensitive hypertension in Dahl SS rats [8].

Work from our laboratory as well as others has linked the development of hypertension with immunity and the increased infiltration of immune cells into the kidney [9-12]. It is also known that NOX2, the phagocytic isoform of NADPH oxidase, is highly expressed on immune cells [13]. Our previous studies have shown that compared to whole kidney homogenates, infiltrating T cells isolated from the kidney highly express the p67<sup>phox</sup> subunit, which becomes exacerbated upon high salt challenge [14]. This is not only true of p67<sup>phox</sup>, but all other subunits of NOX2 as well. These data altogether suggests that renal infiltrating immune cells may be a significant source of ROS, ultimately contributing to disease progression in the Dahl SS rat.

With previous evidence showing greater levels of renal interstitial ROS in Dahl SS rats, and with role of p67<sup>phox</sup> already established in their progression of hypertension and renal damage, the current study sought to investigate the effect of oxidative stress inhibition on salt-induced renal immune cell infiltration. Additionally, we utilized a total body irradiation/ bone marrow transfer (TBI/BMT) technique to dissociate the contribution of NOX2-derived

ROS from immune cells versus the renal parenchyma, and we hypothesize that ROS specifically generated by immune cells is crucial to the development of hypertension and target organ damage in Dahl SS rats.

## Methods

### Animals.

Experiments were performed on age-matched, inbred, male Dahl SS (SS/JrHSDMcwi) and SS<sup>p67phox<sup>-/-</sup></sup> (SS-Ncf2em1Mcwi) rats maintained on a 0.4% NaCl purified casein-based AIN-76A diet (#113755, Dyets Inc). As previously described [8], SS<sup>p67phox<sup>-/-</sup></sup> rats were generated using zinc finger nuclease (ZFN) technology targeting rat *p67phox* exon 2. While studies utilizing female Dahl SS rats are currently ongoing, the exclusive focus on male rats is an important limitation of the current study. All experimental protocols were approved by the MCW Institutional Animal Care and Use Committee.

### Blood pressure measurement and renal damage phenotyping.

At 7 weeks of age, rats were deeply anesthetized with inhalational anesthesia (isoflurane). Using aseptic technique, rats were instrumented with telemetry transmitters (Data Sciences International) into the carotid artery with antibiotic (25mg/kg cefazolin) and analgesia (0.3mg/kg Buprenorphine-SR) administered after surgery. Following a one-week recovery period, baseline blood pressure was measured continuously while the rats were maintained on the 0.4% NaCl purified casein-based diet. At 9 weeks of age, both groups of rats were switched to a high-salt (HS, 4.0% NaCl) diet for 21 days. Urine was collected during the 0.4% NaCl period, and at 7, 14, and 21 days during HS. Urine electrolytes were measured by flame photometry (Model 410, Corning), urinary protein was measured with an autoanalyzer (ACE, Alfa Wasserman), and urinary albumin was quantified with a fluorescent assay that utilized Albumin Blue 580 dye (Molecular Probes) and a fluorescent plate reader (FL600, Bio-Tek).

### Immune cell isolation and flow cytometry.

At the conclusion of the experiment, rats were anesthetized with isoflurane and the kidneys were flushed with heparinized dPBS, minced, and incubated in RPMI 1640 media containing L-glutamine, HEPES, collagenase type IV, and DNase. Mononuclear cells were separated by Percoll density gradient centrifugation, counted on a hemocytometer, and incubated with extracellular markers anti-CD3 (eBioscience), anti-CD4 (BioLegend), and anti-CD8 (BioLegend) for T-cells, anti-CD45R (BD Bioscience) for B-cells, and anti-CD11b/c (eBioscience) for monocytes and macrophages. All cells were analyzed by flow cytometry (LSRII, Becton Dickinson) with FACSDIVA software (Becton Dickinson) and FlowJo software (Tree Star).

### Histology.

Kidney tissues were collected for histological analysis and fixed with 10% neutral buffered formalin, paraffin embedded, cut into 4  $\mu$ m sections, mounted, and stained with Masson's trichrome. The kidney slices were scanned with a Nikon Super CoolScan 9000 interfaced with VueScan x64 software. The outer medullary cast percentage was determined by color

inclusion via MetaMorph Microscopy Automation and Image Analysis software (Molecular Devices).

### **Total body irradiation and bone marrow transfer (TBI/BMT).**

As described previously [15], at 6 weeks of age  $SS^{p67phox-/-}$  rats underwent total body irradiation (TBI) at a dose of 11 Gy at a rate of 1.83 Gy/min. The bone marrow cells from either SS or  $SS^{p67phox-/-}$  were collected from four donor femora and diluted in 6 ml of dPBS. Within 2 hours of TBI, rats received ~0.3 ml dPBS containing either SS or  $SS^{p67phox-/-}$  bone marrow cells via tail vein injection. As described above, two weeks after bone marrow transplantation (BMT), rats were instrumented with telemeters to record blood pressure, switched to HS at 9 weeks of age, and urine collected during the LS period, and at 7, 14, and 21 days during HS. At the conclusion of the experiment, we performed a respiratory burst assay on isolated peritoneal macrophages, followed by tissue harvest, flow cytometric analysis, and histology, as described above.

### **Respiratory burst assay.**

TBI/BMT rats were anesthetized with isoflurane. 10 ml ice-cold 3% FBS in dPBS was injected in to the abdominal cavity followed by a small midline incision to recollect the solution. The collected fluids were centrifuged at 400g for 5 min, followed by incubation with red blood cell lysis buffer (Life Technologies). Similarly performed as previous described [8], Cells were washed, resuspended in Dulbecco's Modified Eagle Medium (Invitrogen), with  $1 \times 10^6$  cells aliquoted into wells in triplicate on a clear-bottom 96-well plate (Bioexpress). The plate was incubated at 37°C for 2 h. Media was then removed and replaced with 0.3 ml of 1 mM luminol derivative L-012 (Wako Pharmaceuticals) dissolved in Hank's balanced salt solution (Invitrogen). Luminescence of L-012 was used as an index of superoxide production. Luminescence was measured at 37°C on FLUOstar Omega machine (BMG Labtech) for baseline recording. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, was added to each well to yield a final concentration of 135µM. Luminescence was then measured every 5 min for 30 min. To determine the specificity of the assay to detect superoxide, superoxide dismutase (100 U/well) was added as a control.

### **Statistical Analysis.**

Data are expressed as the mean  $\pm$  one standard error of the mean. Data were assessed for significance using a t-test, a one-way analysis of variance (ANOVA) with a Holm-Sidak post-hoc test, or a two-way repeated measures ANOVA with a Holm-Sidak post-hoc test, as appropriate.

## **Results**

### **$SS^{p67phox-/-}$ rats demonstrate improved blood pressure, renal damage, and renal immune cell infiltration compared to SS rats.**

Even while both groups were maintained on the 0.4% low-salt diet, the  $SS^{p67phox-/-}$  rats already demonstrated a lower MAP (~11 mmHg) than the SS (Figure 1A). This protection continued through the entire 3 weeks of high salt challenge, where by HS21 the  $SS^{p67phox-/-}$  rats had a 33.2 mmHg lower MAP than the SS rats ( $140.7 \pm 4.8$  vs  $173.9 \pm 8.5$  mmHg,

SS<sup>p67phox<sup>-/-</sup></sup> vs SS). Shown in Figure 1B, this improved blood pressure phenotype in the SS<sup>p67phox<sup>-/-</sup></sup> was mirrored in their protection from renal damage, where the mutant rats also exhibited less urinary protein (179.3±37.3 vs 323.1±40.7 mg/day, SS<sup>p67phox<sup>-/-</sup></sup> vs SS at HS21) and albumin excretion (98.8±19.4 vs 186.3±25.1 mg/day, SS<sup>p67phox<sup>-/-</sup></sup> vs SS at HS21). SS<sup>p67phox<sup>-/-</sup></sup> rats also demonstrated a significant improvement in renal histological damage, seen by a reduction in medullary tubular protein cast formation (4.9±0.8 vs 19.0±2.1 %, SS<sup>p67phox<sup>-/-</sup></sup> vs SS, Figure 2A). Interestingly, examination of immune cells revealed a specific increase in CD3+ T cells within the circulation (Figure 2B). Upon final examination of the immune cells infiltrating the kidney (Figure 2C), there were fewer total CD45+ leukocytes in the kidneys of the SS<sup>p67phox<sup>-/-</sup></sup> compared to SS (4.8±0.3 vs 6.3±0.5 ×10<sup>6</sup> cells/kidney, SS<sup>p67phox<sup>-/-</sup></sup> vs SS), which was primarily attributed to a reduction in CD11b/c+ monocytes and macrophages (3.8±0.2 vs 5.2±0.5 ×10<sup>6</sup> cells/kidney), as well as CD45R+ B cells (1.4±0.3 vs 2.6±0.3 ×10<sup>5</sup> cells/kidney). Interestingly, there was no difference in the infiltration of CD3+ T cells. These data altogether demonstrated the importance of NOX2-derived ROS in the development of SS hypertension, renal damage, and renal immune cell infiltration.

### **Respiratory burst assay in isolated peritoneal macrophages from TBI/BMT rats demonstrated the ability of SS-derived hematopoietic cells to produce ROS.**

In order to delineate between the contribution of ROS from the renal parenchyma versus ROS from infiltrating immune cells, we utilized a total body irradiation/bone marrow transfer (TBI/BMT) approach ablating the resident bone marrow cells from SS<sup>p67phox<sup>-/-</sup></sup> host rats which then received bone marrow from either an SS or SS<sup>p67phox<sup>-/-</sup></sup> donor rat. With both groups utilizing SS<sup>p67phox<sup>-/-</sup></sup> as the host, neither express p67<sup>phox</sup> in any of their tissue. However, with the host hematopoietic cells repopulating from the donor, the SS<sup>p67phox<sup>-/-</sup></sup> host receiving bone marrow from an SS donor should have intact p67<sup>phox</sup> in all cells derived from the hematopoietic cell lineage. We would expect these SS donor cells to produce NOX2-derived ROS in their immune cells, while the cells from the SS<sup>p67phox<sup>-/-</sup></sup> donor would not. As proof of principle, we performed a respiratory burst assay in peritoneal macrophages isolated from rats receiving TBI/BMT to see if these immune cells derived from the reconstituted SS bone marrow were indeed functional. Shown in Figure 3, unstimulated control macrophages from SS donors displayed a higher superoxide production baseline compared to those isolated from SS<sup>p67phox<sup>-/-</sup></sup> donors (1.9-fold higher at 30 min). Upon stimulation with PKC activator and ROS generator phorbol myristate acetate (PMA), there is a striking increase in superoxide detected in the macrophages from SS donors (10.2-fold higher at 30 min), which is not evident in the stimulated macrophages from SS<sup>p67phox<sup>-/-</sup></sup> donors. To assess specificity of the signal for the detection of superoxide, a separate set of stimulated samples were co-incubated with superoxide dismutase which demonstrated levels even lower than baseline, indicating additional sources of O<sub>2</sub><sup>-</sup> other than NOX2. These results critically demonstrate the functionality and ability of immune cells reconstituted from the SS donor bone marrow to produce ROS.

## NADPH oxidase-derived ROS production in hematopoietic cells is necessary for the development of salt-sensitive hypertension and renal damage in SS rats.

There was no difference in 0.4% NaCl baseline blood pressure between SS<sup>p67<sup>phox</sup>-/-</sup> rats receiving bone marrow from either SS or SS<sup>p67<sup>phox</sup>-/-</sup> (Figure 4A). Upon high salt challenge, SS recipients (+SS) demonstrated a clear and significant exacerbation of hypertension compared to SS<sup>p67<sup>phox</sup>-/-</sup> recipients (+SS<sup>p67<sup>phox</sup>-/-</sup>) (176.1±4.7 vs 147.9±8.4 mmHg, +SS vs +SS<sup>p67<sup>phox</sup>-/-</sup> at HS21). This exacerbated blood pressure phenotype in the +SS group was accompanied with greater renal damage evident by more proteinuria (287.2±25.1 vs 179.5±24.6 mg/day, +SS vs +SS<sup>p67<sup>phox</sup>-/-</sup> at HS21) and albuminuria (168.3±23.7 vs 107.0±20.4 mg/day, +SS vs +SS<sup>p67<sup>phox</sup>-/-</sup> at HS21) (Figure 4B). At the conclusion of the study, there was greater renal histological damage in the +SS rats, evident by the significant percentage of protein casts found in the outer medulla of SS donor rats (33.2±4.7 vs 8.1±3.5 %, +SS vs +SS<sup>p67<sup>phox</sup>-/-</sup>, Figure 5A). Inspection of the immune cell subsets found in the circulation revealed no change between +SS and +SS<sup>p67<sup>phox</sup>-/-</sup> (Figure 5B), although the total number of circulating immune cells is lower in these TBI/BMT rats compared to non-irradiated rats (Figure 2B), indicative of an incomplete reconstitution of bone marrow cells. Despite this reduced peripheral reconstitution, examination of the infiltrating renal immune cells primarily showed no difference between the two groups, though there was trending increase in CD11b/c+ monocytes and macrophages in the kidneys of +SS rats (4.7±0.4 vs 3.5±0.5 ×10<sup>6</sup> cells/kidney, +SS vs +SS<sup>p67<sup>phox</sup>-/-</sup>, p=0.06, Figure 5C). With the only difference between the +SS and +SS<sup>p67<sup>phox</sup>-/-</sup> being the ability to produce NOX2-derived ROS in the hematopoietic cells, this suggests that ROS production in immune cells is important for the development and progression of salt-induced hypertension and renal damage, while not significantly altering the homing of immune cells to the kidney.

## Discussion

The present study demonstrated that inhibition of NADPH oxidase via p67<sup>phox</sup> mutation in Dahl SS rats (SS<sup>p67<sup>phox</sup>-/-</sup>) ameliorates salt-induced hypertension, renal injury, and renal immune cell infiltration compared to SS controls. Additionally, transfer of functional, ROS-producing SS bone marrow cells into irradiated SS<sup>p67<sup>phox</sup>-/-</sup> worsened disease, indicating that oxidative stress specifically generated by immune cells is sufficient for the exacerbation of hypertension and target organ damage in Dahl SS rats.

Though the importance of NOX2 NADPH oxidase was originally discovered through its association with chronic granulomatous disease, its role in immunity and disease has now been vastly expanded to a whole host of pathologies, including cardiovascular and renal disease [16, 17]. In the Dahl SS rat, high salt challenge resulted in elevated levels of intrarenal ROS (O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>) and hypertension [3, 7]. As evidence for the specific contribution of NOX2, genetic mutation of the p67<sup>phox</sup> subunit in SS rats via zinc finger nuclease technology reduced salt-sensitive hypertension, reversed reductions in medullary blood flow, maintained GFR, and consequently prevented renal injury [14, 18]. It is worth noting that p67<sup>phox</sup> has also been demonstrated to also support the activity of both NOX1 and NOX3 [19, 20], though NOX1 is most highly expressed in the colon and NOX3 in the adrenal gland. Aside from increases in NADPH oxidase activity, increased oxidative stress



and accelerated disease progression have also been attributed to deficiencies in antioxidants like superoxide dismutase [21, 22]. NADPH oxidase inhibition or antioxidant treatment has been widely demonstrated to have beneficial effects in multiple preclinical hypertension and renal disease settings, either specifically in the Dahl SS rat [2, 3, 7, 23, 24] or in other experimental models [25-29], and is the current target of potential therapeutic agents in ongoing clinical trials.

It is well established that the presence of immune cells in target organs is an important mechanism underlying the development of hypertension and renal injury [30]. While NADPH oxidases in various tissues, including the kidney, have been thoroughly characterized [13, 31], the contribution of ROS derived from either resident or infiltrating immune cells in the kidney remain unknown. NOX2, also known as phagocytic NADPH oxidase, is highly expressed on macrophages/monocytes and granulocytes, and since these CD11b/c+ cells comprise the majority of the total CD45+ leukocytes in the kidney (>80%, Figure 2), it is highly plausible that these renal immune cells can play a pivotal role in determining the oxidant status of the kidney. Furthermore, there is a substantial body of evidence highlighting the integral role of oxidative stress in the activation of the immune system and the progression of hypertensive diseases [32]. This is especially true for NOX2-derived ROS, which has been demonstrated to regulate a wide number of immunological processes related to chemotaxis, monocyte and macrophage infiltration, innate inflammasome activation, antigen cross-presentation, adaptive immune activation, and T cell selection, maturation, and differentiation [33-38]. Dysregulation of these key immune mechanisms may serve as an explanation as to how oxidative imbalance could lead to inappropriate immune activation, thus leading to target organ damage and subsequent hypertension.

The current study revealed through a TBI/BMT approach that the production of NOX2-derived ROS solely in cells of hematopoietic origin (immune cells), but absent in the remainder of the host, was sufficient to drive and exacerbate salt-induced hypertension, renal damage, and immune cell infiltration, highlighting the fundamental role of immune cell ROS in the pathogenesis of Dahl SS disease. More crucially, these results demonstrate the importance of the exact cellular localization and source of ROS production, which becomes critical when determining targets for potential therapeutic applications. Despite extensive, strong pre-clinical data, many clinical trials have failed to provide evidence in support of antioxidant therapy for a number of various diseases [39, 40], for reasons primarily related to short trial duration and poor antioxidant bioavailability [41, 42]. In light of the current study, perhaps taking a more targeted approach to deliver high enough concentrations of antioxidants to the cells specifically responsible for producing injurious ROS would lead to more efficacious therapies and ultimately better outcomes in humans.

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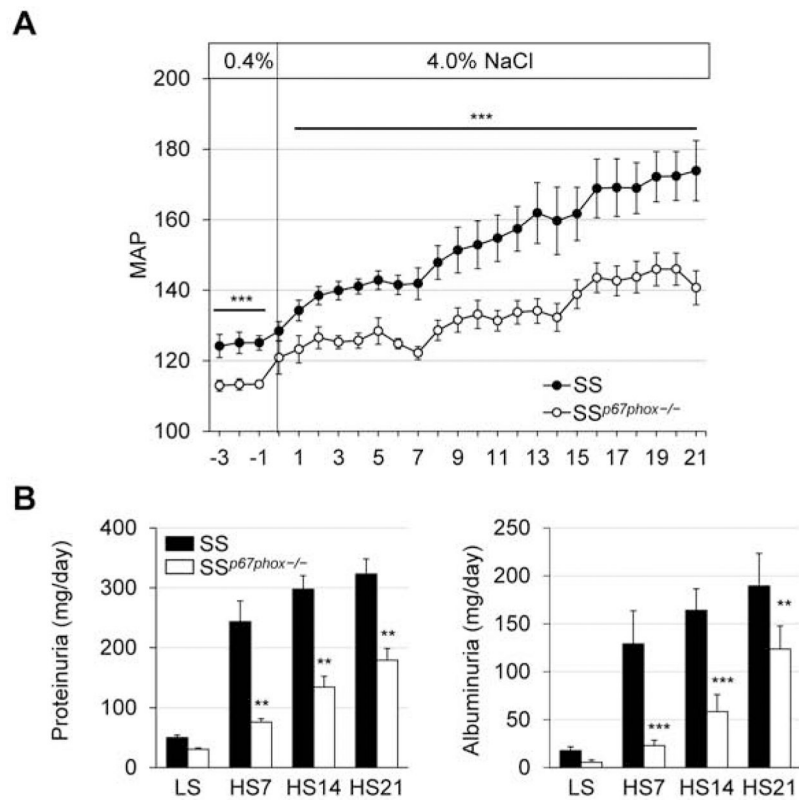
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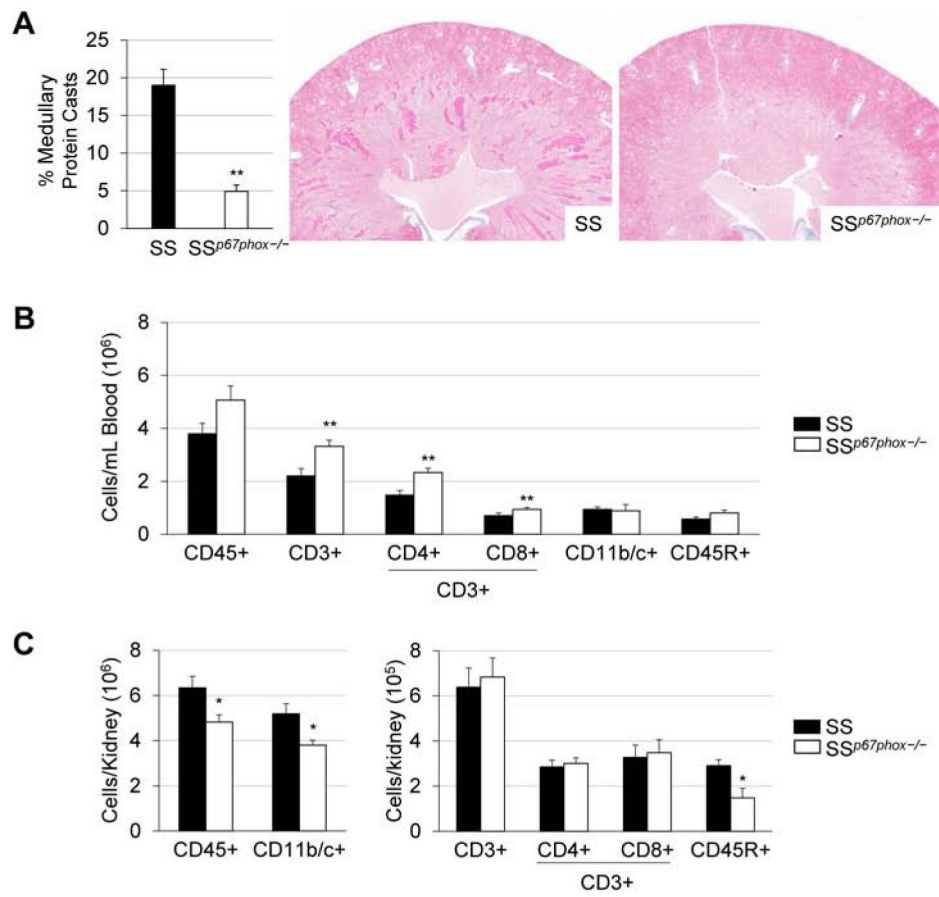
### Highlights

- Aberrant ROS production has been implicated in the development of salt-sensitive (SS) hypertension and renal damage
- Inhibition of NOX2 ROS via p67<sup>phox-/-</sup> in Dahl SS rats lowered blood pressure and improved renal damage and inflammation
- A total body irradiation/bone marrow transfer approach was utilized to assess the specific contribution of ROS derived from immune cells
- Immune cell production of NOX2-derived ROS sufficiently exacerbates SS hypertension, renal damage, and renal inflammation



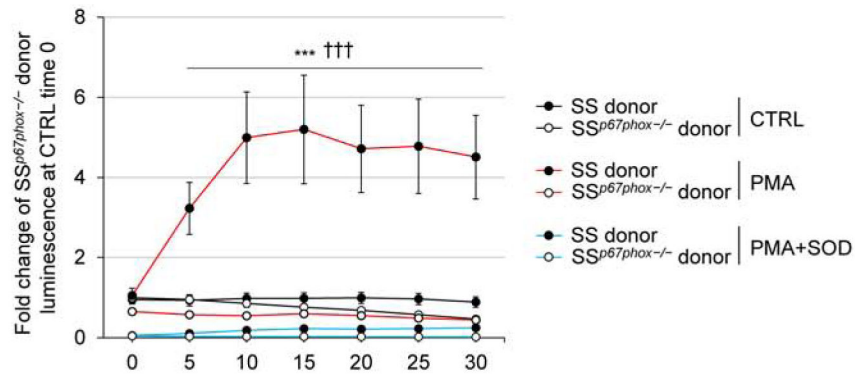
**Figure 1. Attenuation of hypertension and renal damage in SS<sup>p67phox-/-</sup> rats.**

(A) Daily mean arterial pressure (MAP) averages over the course of 3 weeks of 4.0% NaCl challenge. n=7-8. (B) Weekly determination of renal damages indices, urinary protein and albumin excretion. n=8-9. \*\*p<0.005, \*\*\*p<0.001 SS<sup>p67phox-/-</sup> vs SS.



**Figure 2. Attenuation of renal histological damage and renal immune cell infiltration in SS<sup>p67phox-/-</sup> rats.**

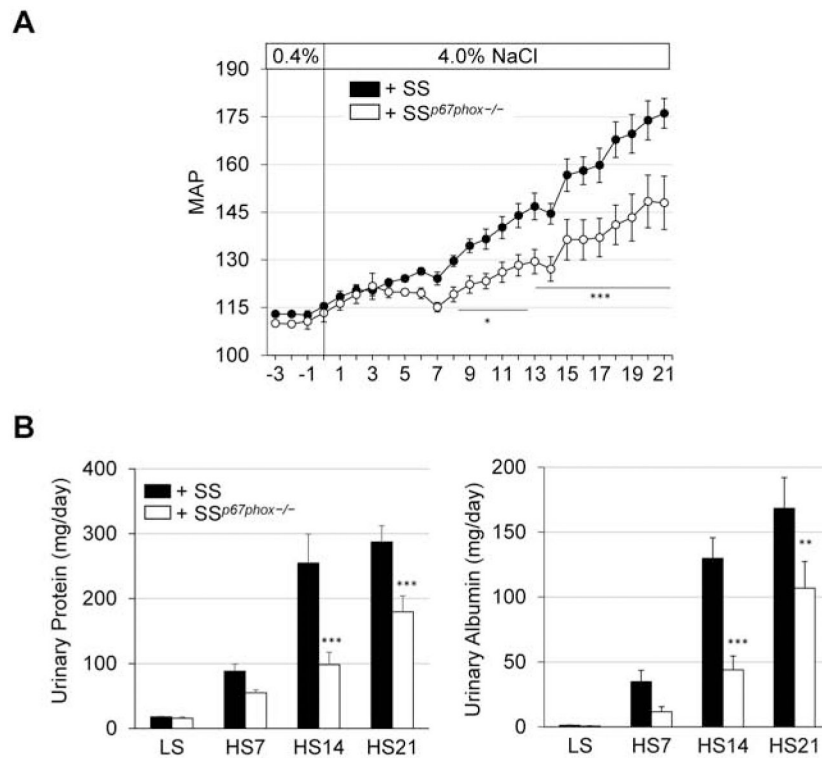
(A) Percentage of medullary protein cast formation in the kidneys of SS and SS<sup>p67phox-/-</sup> rats after 3 weeks of 4.0% NaCl challenge. n=7-9. (B) Circulating immune cell profile between SS and SS<sup>p67phox-/-</sup> rats. (C) Absolute numbers of immune cells infiltrating into the kidneys at the conclusion of the experiment. CD45+: leukocytes, CD11b/c+: monocytes/macrophages, CD3+: T cells, CD4+: T-helper cells, CD8+: cytotoxic T cells, CD45R+: B cells. n=7-9. \*p<0.05, \*\*p<0.01 SS<sup>p67phox-/-</sup> vs SS.



**Figure 3. Respiratory burst assay in isolated peritoneal macrophages from TBI/BMT rats demonstrates the ability of SS-derived hematopoietic cells to produce ROS.**

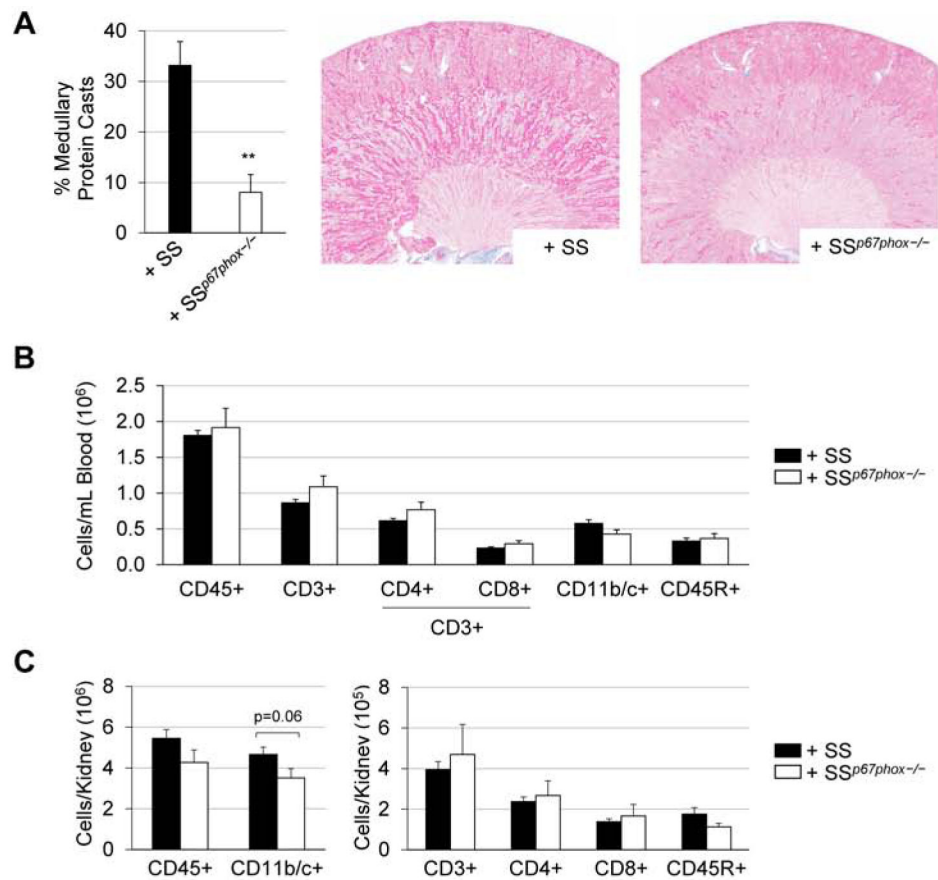
Luminol derivative L-012 was used as an index of superoxide production. Superoxide production was detected in PMA-stimulated macrophages from isolated from rats received bone marrow from SS donors, which was not evident in macrophages from SS<sup>p67phox</sup>-/- donors.  $1 \times 10^6$  peritoneal macrophages/well, PMA = 135  $\mu$ M, SOD = 100 U/well. n=6-8.  $P_{\text{Group}} < 0.001$  SS<sup>p67phox</sup>-/- vs SS,  $***p < 0.001$  PMA vs Ctrl (SS),  $\dagger\dagger\dagger p < 0.001$  PMA vs PMA+SOD (SS).





**Figure 4. NADPH oxidase-derived ROS production in immune cells is necessary for the development of salt-sensitive hypertension and renal damage in SS rats.**

(A) Exacerbated rise in mean arterial pressure (MAP) in SS<sup>p67phox-/-</sup> receiving SS bone marrow (+SS) after 3 weeks of 4.0% NaCl challenge. n=4-5. (B) Renal damage indices, urinary protein and albumin excretion, were also worsened in rats receiving SS bone marrow (+SS) compared to those receiving SS<sup>p67phox-/-</sup> bone marrow (+SS<sup>p67phox-/-</sup>). n=9-11. \*p<0.05, \*\*p<0.005, \*\*\*p<0.001 +SS<sup>p67phox-/-</sup> vs +SS.



**Figure 5. Exacerbation of renal histological damage in SS<sup>p67phox-/-</sup> receiving SS bone marrow (+SS) rats.**

(A) Percentage of medullary protein cast formation in the kidneys of +SS and +SS<sup>p67phox-/-</sup> rats after 3 weeks of 4.0% NaCl challenge. n=7-9. (B) Circulating immune cell profile between +SS and +SS<sup>p67phox-/-</sup> rats. (C) Absolute numbers of immune cells infiltrating into the kidneys at the conclusion of the experiment. CD45+: leukocytes, CD11b/c+: monocytes/macrophages, CD3+: T cells, CD4+: T-helper cells, CD8+: cytotoxic T cells, CD45R+: B cells. n=9-10. \*p<0.05, \*\*p<0.01 +SS<sup>p67phox-/-</sup> vs +SS.