Original Article

Dendritic cells are crucial for cardiovascular remodeling and modulate neutrophil gelatinase-associated lipocalin expression upon mineralocorticoid receptor activation

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Background: Adaptive immunity is crucial in cardiovascular and renal inflammation/fibrosis upon hyperactivation of mineralocorticoid receptor. We have previously demonstrated that dendritic cells can respond to mineralocorticoid receptor activation, and the neutrophil gelatinase-associated lipocalin (NGAL) in dendritic cells is highly increased during aldosterone (Aldo)/mineralocorticoid receptor-dependent cardiovascular damage. However, the interrelationship among dendritic cells, target organs inflammation/fibrosis induced by mineralocorticoid receptor, and NGAL-dependence remains unknown.

Objective: We studied the role of dendritic cells in mineralocorticoid receptor-dependent tissue remodeling and whether NGAL can modulate the inflammatory response of dendritic cells after mineralocorticoid receptor activation.

Methods: Cardiovascular and renal remodeling induced by Aldo and high-salt diet [nephrectomy-Aldo-salt (NAS) model] were analyzed in CD11c.DOG mice, a model which allows dendritic cells ablation by using diphtheria toxin. In addition, in-vitro studies in NGAL-knock out dendritic cells were performed to determine the immunomodulatory role of NGAL upon Aldo treatment.

Results: The ablation of dendritic cells prevented the development of cardiac hypertrophy, perivascular fibrosis, and the overexpression of NGAL, brain natriuretic peptide, and two profibrotic factors induced by NAS: collagen 1A1 and connective tissue growth factor. We determined that dendritic cells were not required to prevent renal hypertrophy/fibrosis induced by NAS. Between different immune cells analyzed, we observed that NGAL abundance was higher in antigen-presenting cells, while in-vitro studies showed that mineralocorticoid receptor stimulation in dendritic cells favored NGAL and IL-23 expression (p19 and p40 subunits), which are involved in the development of fibrosis and the Th17-driven response, respectively.

Conclusion: NGAL produced by dendritic cells may play a pivotal role in the activation of adaptive immunity that leads to cardiovascular fibrosis during mineralocorticoids excess.

Keywords: cardiovascular fibrosis, dendritic cells, inflammation, mineralocorticoid receptor, neutrophil gelatinase-associated lipocalin

Abbreviations: APC, antigen-presenting cell; BM-DC, bone marrow-derived dendritic cell; Col1A1, collagen 1A1; IL-23p19, IL-23, p19 subunit; IL-23p40, IL-23, p40 subunit; MHC, major histocompatibility complex; NAS, nephrectomy–aldosterone–salt; NGAL, neutrophil gelatinase-associated lipocalin; PD-L1, programmed death-ligand 1; RORγ, Tthymus-specific RAR-related orphan receptor gamma

INTRODUCTION

Hyperactivation of the mineralocorticoid receptor and high-salt intake promote hypertension and a proinflammatory state, leading to tissue

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remodeling, and cardiovascular fibrosis [1]. It has been demonstrated that the activation of immune system is crucial for hypertension induced by alterations of the renin–angiotensin–aldosterone (Aldo) system (RAAS) [2] and mineralocorticoid receptor activation [3]. Indeed, T cells [4] and $CD11c^+$ dendritic cells [5], both key players for the adaptive immune response, are required to induce RAAS-dependent high blood pressure. In our previous works, we have reported that mineralocorticoid receptor activation on dendritic cells can promote Th17 lymphocytes (Th17, a subset of $CD4^+$ T cells) [6] mediated immunity, and that mineralocorticoid receptor blockade in hypertensive rats treated with deoxycorticosterone acetate and high-salt diet (DOCA-salt) dampens the Th17 response in heart, kidney, and peripheral blood by modulating regulatory T cells [7]. Although these results suggest a critical role of mineralocorticoid receptor-signaling on dendritic cells and/or T cells contributing to cardiac and renal inflammation/fibrosis during high blood pressure, they do not provide information concerning the mechanisms downstream mineralocorticoid receptor activation.

The carrier protein neutrophil gelatinase-associated lipocalin (NGAL), also named lipocalin 2, is a secreted protein of 25 kDa belonging to the lipocalin superfamily able to bind siderophores [8,9]. NGAL is found in kidney [10], diverse cells types in cardiovascular system [11], and in hematopoietic organs with relatively high abundance [12]. NGAL has been identified as a marker closely associated with cardiovascular and metabolic diseases [13], and as sensitive biomarker in response to renal ischemic injury [14] and nephrotoxicity [15]. It has been demonstrated that plasma NGAL levels correlate positively with the mean arterial pressure in essential hypertensive patients, suggesting its participation in cardiovascular disease [16]. Moreover, Aigner *et al.* [12] showed that genetic deficiency of NGAL abolished the recruitment of immune cells into the heart upon ischemia-reperfusion. This was associated with a lower level of chemokines [17] and decreased adhesion of proinflammatory cells [18].

At the molecular level, NGAL has been identified as a specific target of mineralocorticoid receptor signaling in cardiovascular cells: NGAL expression is increased after Aldo treatment in cardiac, aortic endothelial, and vascular smooth muscle cells [9,11]. Gilet *et al.* [19] demonstrated that Aldo-dependent mineralocorticoid receptor activation induced the formation of NGAL complex with matrix metalloprotease-9 in human neutrophils, a marker of neutrophil activation during inflammatory responses and tissue remodeling. Recently, we showed that not only whole genetic deficiency of NGAL, but also the specific depletion in immune cells attenuates the cardiovascular fibrosis and proinflammatory phenotype induced by Aldo infusion and a high-salt diet in uninephrectomized mice (nephrectomy–Aldo–salt, NAS model) [20,21].

However, the specific immune cell type responsible for NGAL production, and its impact on hypertension, inflammation and cardiovascular/renal remodeling upon mineralocorticoid receptor activation remain unknown. As dendritic cells orchestrate the adaptive immunity in chronic inflammation, we hypothesized that dendritic cells are relevant during high blood pressure and cardiovascular fibrosis in NAS mice, and that NGAL plays a pivotal role in dendritic cells as an immune modulator upon mineralocorticoid receptor activation.

MATERIALS AND METHODS

Animals and in-vivo experimental protocol

For in-vivo studies, we used 16-week-old females CD11c.DOG mice [20], a mouse model that use the CD11c promoter to express a diphtheria toxin receptor transgene to specifically deplete $CD11c^{high}$ dendritic cells [22]. Hochweller *et al.* [22] have previously demonstrated that the diphtheria toxin administration to CD11c.DOG mice decrease $CD11c^{hi}$ cells by more than 95%, which was corroborated by us at 48 h after the first injection (Fig. S1, http://links.lww.com/HJH/B72). For in-vitro studies, we used 6–8-week-old males C57BL/6 wild-type mice and constitutively inactivated for NGAL mice (NGAL-knock out) [21]. Animal breeding, housing, and protocols were performed in accordance with the ethical guidelines of Institut National de la Santé et de la Recherche Médicale for the care and use of laboratory animals. Our local ethical committees for animal experimentations approved all experiments. All animal experimentations adhere to the National Institute of Health (NIH) Guide for Care and Use for the Laboratory Animals. The animals were treated according to the experimental protocol described below.

CD11c.DOG mice were randomized in three groups ($n = 4–5$ per group): first, DOG Vehicle (diphtheria toxin−) group, where mice received intraperitoneal injection of 100 μl of PBS 1× as vehicle (control), second, DOG NAS (diphtheria toxin−) group, where uninephrectomized mice were challenged with NAS (Aldo, 500 μg/kg per day + 1% NaCl in drinking water) and intraperitoneal injection PBS 1×, and third, DOG NAS (diphtheria toxin+) group, where mice where challenged with NAS (Aldo, 500 μg/kg per day) and intraperitoneal injection diphtheria toxin (8 ng/g per day) (Sigma-Aldrich, St Louis, Missouri, USA) to induce depletion of dendritic cells. All mice were sacrificed 14-days later.

Physiological studies

SBP was measured routinely by tail-cuff plethysmography in trained conscious mice. Data were recorded using PowerLab Chart five and analyzed with LabChart Reader software (ADInstruments, Colorado Springs, Colorado, USA). The data of SBP that we present was obtained at day 14. At the same time, plasma was collected and stored at −20 °C. Plasma creatinine was analyzed by using automated chemistry (Jaffe Assay). Tissues were quickly removed, weighed, and used for histological and biochemical analyses.

Histological analysis

Sagittal sections of kidneys and transversal sections of hearts were fixed in 10% formalin, embedded in paraffin and 5-μm tissue sections were prepared using a Leica Manual Microtome Type 1212 (Leica Biosystems, Wetzlar, Germany). The sections were washed and stained with Masson's Trichrome for the assessment of tissue fibrosis. Also, equatorial ventricular sections were stained with

hematoxylin-eosin to determine cardiomyocyte cross-sectional area. Images were captured with a MicroPublisher 3.3 RTV digital camera (QIMAGING, Surrey, British Columbia, Canada) attached to an Olympus CX31 microscope (Olympus Corp., Tokyo, Japan). Images were analyzed and scored by a blinded method, using MShot Digital Imaging System software (Microshot Technology, Guangzhou, China).

Dendritic cells differentiation and treatments

Bone marrow-derived dendritic cells (BM-DCs) from wild-type and NGAL-knockout mice from 6 to 8-week-old males were prepared as previously described [6]. Briefly, femurs, and tibias were cleaned and flushed out through bone cavity with sterile RPMI 1640 (Hyclone, Logan, Utah, USA). Collected cells were pelleted by centrifugation [1800 rpm for 6 min at room temperature (RT)], and suspended in a differentiation RPMI 1640 supplemented with 10% heat-inactivated FBS (Biological Industries, Beit Haemek, Israel) FBS medium and 10 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) for dendritic cells differentiation[21] (Peprotech, Rocky Hill, New Jersey, USA). The culture media was replaced each 2–3 days. On day 7, differentiation of dendritic cells was routinely assessed obtaining more than 80% $CD11c^+$ cells. After 7-days of differentiation, medium was replaced for fresh RPMI 1640 5% charcoal stripped FBS medium and, 24 h later (day 8), adhesives dendritic cells were stimulated with Aldo (100 nmol/l) or vehicle during 24 h, Spironolactone 5 μmol/l. As a positive control, dendritic cells were treated with 1 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich). Mouse recombinant NGAL (500 ng/ml; R&D Systems, Minneapolis, Minnesota, USA) during 18 h was used for further experiments.

To determine expression levels of key surface molecules, dendritic cells were immunostained with the following Abs: antigen-presenting cell (APC) anti-CD11c (clone N418), Peridinin-chlorophyll-protein (PerCP) antimajor histocompatibility complex (MHC)-II (clone M5/114.15.2), FITC-anti-CD80 (clone 16–10A1), PE-CD86 anti-CD86 (clone GL1), and PE-Cy7-antiprogrammed death-ligand 1 (PD-L1) (clone 10F.9G2), all of them from Biolegend.

T-cell coculture with dendritic cells

Control or Aldo-pulsed dendritic cells were washed and resuspended in fresh prewarmed medium. Subsequently, dendritic cells were cocultured at 1 : 2 ratios with either purified OT-I or OT-II T cells (10^5 T cells/well) in the presence of 0.1 ng/ml $OVA_{257-264}$ peptide or 200 ng/ml $OVA_{323-339}$ peptide (GenScript, Piscataway, New Jersey, USA), respectively. Purification of OT-I and OT-II T cells from total splenocytes was carried out by negative selection using $CD8^+$ or $CD4^+$ T cells isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. For determination of T cells proliferation and differentiation, purified OT-I or OT-II T cells were stained with 5 mmol/l Cell Trace Violet (CTV, Invitrogen, Carlsbad, California, USA) according to manufacter's instructions. Subsequently, cells were washed and cocultured with dendritic cells as indicated earlier. After 72 and 120 h of coculture incubation, cells were restimulated with 1 mg/ml ionomycin and 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 h in the presence of 5 mg/ml brefeldin A. For intracellular cytokine staining analysis, cells were first stained either with PerCP anti-CD8 (clone 53-6.7) or PerCP anti-CD4 (clone RM4-5) and PE anti-TCRβ (clone H57-597) and then intracellular staining was done with the FoxP3 staining buffer set (eBioscience, San Diego, California, USA) and the following abs from Biolegend: APC-Cy7 anti-IL-17 (clone TC11-18H10.1), PE-Cy7 anti-IFN-γ (clone XMG1.2), APC anti-Tbet (clone 4B10) and from eBioscience: PE antithymus-specific RAR-related orphan receptor gamma (RORγT) (clone AFKSJS-9). CTV-associated fluorescence and frequency of cytokine-producing T cells were analyzed by flow cytometry in a FACSCanto (BD Biosciences, San Jose, California, USA) and analyzed with FlowJo software (Tree Star, Yorba Linda, California, USA).

Cell sorting flow cytometry

For cell sorting experiment, whole spleen from control wild-type mice were harvested, pooled and dispersed in PBS 1× at 4 °C, and filtered through 40 μm nylon strainer (BD Biosciences). Red blood cells were lysed with ACK lysis buffer. Cells were collected by centrifugation (1800 rpm for 6 min at RT), washed in PBS 1× 2% FBS, and stained for macrophages, dendritic cells, B lymphocytes, T-$CD4^+$, and T-$CD8^+$ lymphocytes, by using the following antibodies: B220-FITC (clone RA3-6B2), CD3-PE (clone 145-2C11), CD4-PeCy5 (clone RM4-5), CD8-APC (clone 53-6.7), CD11b-APC-Cy7 (clone M1/70), CD11c-PE-Cy7 (clone N418), and F4/80-PE-Cy5 (clone BM8). Fluorescence data from 1×10^6 events were sorted from the live gate using a FACSAria II (BD Bioscience). After separation, the purity of cell populations was verified and certified with a value more than 94% in all cases. All data were processed and analyzed using FACSDiva Software (BD Bioscience).

Total RNA isolation and real-time-PCR

Total RNA from heart and kidney, sorted cells and dendritic cells in culture, was extracted with TRIzol according to manufacturer's instructions. cDNA was produced from 2 μg of RNA using Superscript II reverse transcriptase kit. Real-time PCR reactions were performed, and transcript levels were detected by SYBR Green method. The following primers were used: *18S*, (F) 5′-CGCCGCTAGAGGTGAAATTC-3′, (R) 5′-TCTTGGCAAATGCTTTCGC-3′; *BNP,* (F) 5′-CAGCTCTTGAAGGACCAAGG-3′; (R) 5′-AGACCCAGGCAGAGTCAGAA-3′; *Col1a1*, (F) 5′-GAGCGGAGAGTACTGGATCG-3′, (R) 5′-TACTCGAACGGGAATCCATC-3′; *CTGF*, (F) 5′-TGACCCCTGCGACCCACA-3′, (R) 5′-TACACCGACCCACCGAAGACACAG-3′; *GAPDH*, (F) 5′-TCCGTGTTCCTACCCCCAATG-3′, (R) 5′-GAGTGGGAGTTGCTGTTGAAG-3′; IFN-γ (F) 5′-GGTGCAACTGGGATCGCTACATAA-3′, (R) 5′-AGAGCCTGATTCCTTTCCTTCCCT-3′; *IL-6*, (F) 5′-AGGATACCACTCCCAACAGACCT-3′, (R) 5′-CAAGTGCATCATCGTTGTTCATAC-3′; *IL-17A*, (F) 5′-CAAACACTGAGGCCAAGGAC-3′, (R) 5′-TTTCCCTCCGCATTGACAC-3′; *NGAL*, (F) 5′-GGACCAGGGCTGTCGCTACT-3′, (R) 5′-GGTGGCCACTTGCACATTGT-3′; *p19 Subunit*, (F) 5′-CCAGCGGGACATATGAATCT-3′, (R) 5′-AGGCTCCCCTTTGAAGATGT-3′; *p40 Subunit*, (F) 5′-ACAGCACC-

AGCTTCTTCATCAG-3′, (R) 5′-TCTTCAAAGGCTTCATC-TGCAA-3′; *TGF-β1*, (F) 5′-TGCGCTTGCAGAGATTAAAA-3′, (R) 5′-CTGCCGTACAACTCCAGTGA-3′. All PCR products were subjected to melting curve program to confirm amplification specificity. Results were analyzed according to the standard curve method, and mRNA abundance was calculated to the relative amount of 18S or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for each sample.

Statistics

Results from in-vivo and in-vitro studies were expressed as mean ± SEM. Data were analyzed by 1-ways or 2-ways analysis of variance (ANOVA) test followed by Bonferroni post-hoc test (> two groups), or by Mann–Whitney non-parametric test (two groups). All analyses were performed using GraphPad Prism V6.01 (GraphPad Software, San Diego, California, USA). Values of P less than 0.05 were considered as statistically significant.

RESULTS

Dendritic cells depletion attenuates the development of nephrectomy–aldosterone–salt-induced cardiac hypertrophy and fibrosis

To study the involvement of dendritic cells in cardiac fibrosis development upon mineralocorticoid challenge *in vivo*, we used the CD11c.DOG mice (DOG-mice) model undergoing the NAS challenge. As expected, NAS induced an increase in blood pressure and cardiac hypertrophy, as shown by the increase in cardiac weight/tibia length ratio at day 14; these effects were absent in dendritic cells-depleted DOG-mice (diphtheria toxin+ group) (Fig. 1a). These results were consistent with an increase cross-sectional area of cardiomyocytes of DOG NAS (diphtheria toxin−) mice in comparison with DOG-vehicle (diphtheria toxin−) (290.8 ± 33.5 and $177.1 \pm 8.4\,\mu m^2$, respectively), an effect prevented in DOG-NAS (diphtheria toxin+) mice ($176.7 \pm 14.0\,\mu m^2$) (Fig. 1b). Furthermore, NAS treatment induced perivascular fibrosis in coronary vessels of DOG-vehicle (diphtheria toxin−) mice, but not in DOG-NAS (diphtheria toxin+) mice ($P < 0.05$, Fig. 1c).

At the molecular level, transcripts for NGAL, brain natriuretic peptide and for two additional profibrotic mediators, collagen-1 (Col1A1) and connective tissue growth factor, were increased by the NAS challenge in the heart of DOG-NAS (diphtheria toxin−) mice. This effect was prevented by the depletion of dendritic cells in the DOG-NAS (diphtheria toxin+) mice (Fig. 1d). As our previous studies showed Th17-driven responses induced by DOCA-salt in rats, we next analyzed cardiac IL-17A transcript after mineralocorticoid receptor activation in NAS mice. We found an increase of IL-17A mRNA abundance in DOG-NAS (diphtheria toxin−) mice ($P < 0.05$), however, this effect was not prevented in dendritic cells-depleted DOG-mice (diphtheria toxin+ group) (Fig. S3A, http://links.lww.com/HJH/B72). As a marker for Th1-driven response [23], we evaluated IFN-γ transcript, but we did not observe any difference between groups (Fig. S3B, http://links.lww.com/HJH/B72).

Dendritic cells deficiency does not prevent the renal dysfunction neither renal hypertrophy nor fibrosis induced by nephrectomy–aldosterone–salt

NAS treatment induced an increase of plasma creatinine and renal hypertrophy after 14 days; dendritic cells depletion (NAS diphtheria toxin+) only attenuated the induction of plasma creatinine (Fig. 2a and b). In addition, we observed induction of renal fibrosis in the DOG-NAS (diphtheria toxin−) group (1.12 ± 0.057 vs. 1.93 ± 0.17 arbitrary units), which was not abolished by dendritic cells ablation in the DOG-NAS (diphtheria toxin+) (1.55 ± 0.31 arbitrary units, Fig. 2c). These effects were associated with a renal increase of NGAL and Col1A mRNA abundance, after NAS treatment (Fig. 2d). The absence of dendritic cells did not prevent these changes.

We observed that renal IL-17A and IFN-γ mRNA abundance were up regulated by NAS treatment, however, only IFN-γ was significantly decreased in NAS diphtheria toxin+ mice, ($P < 0.05$, Fig. S3A and B, http://links.lww.com/HJH/B72). In addition, we determined that NAS treatment did not increase any of markers of dendritic cells in the heart (Fig. S4A and B, http://links.lww.com/HJH/B72). Only dendritic cells maturation markers, such as MHC-II and CD86, were increased at the mRNA level in kidney after NAS treatment (Fig. S4C, http://links.lww.com/HJH/B72).

Neutrophil gelatinase-associated lipocalin is mainly expressed in antigen-presenting cells and is further upregulated after mineralocorticoid receptor activation in dendritic cells

As dendritic cells were critical in cardiac hypertrophy, fibrosis and NGAL overexpression after NAS treatment, we characterized NGAL expression in dendritic cells and other immune cells described to be relevant in the inflammatory response during hypertension related to mineralocorticoids and RAAS-hyperactivation [3–5,24]. Dendritic cells (MHC-class-II$^+$CD11c^{high}CD11b$^-$), macrophages (CD11b$^+$F4/80$^+$CD11c$^-$), B cells (B220$^+$ CD11b$^-$), CD4 (CD3$^+$CD4$^+$), and CD8 (CD3$^+$CD8$^+$) T cells were isolated by cell sorting flow cytometry from the spleen of wild-type mice, while the whole kidney was used as reference for NGAL expression. Particularly, APC, including macrophages and dendritic cells, presented the highest levels of NGAL mRNA, while B cells, CD4$^+$ and CD8$^+$ T cells showed lower NGAL relative abundance (Fig. 3a).

In a recent study, we showed that NGAL expression in immune cells is crucial for cardiovascular fibrosis induced by the mineralocorticoid receptor activation [20]. Here, our results strongly suggest that dendritic cells are necessary for the development of cardiac hypertrophy and fibrosis induced by NAS. As dendritic cells express high levels of NGAL, we also wanted to study the direct effect of mineralocorticoid receptor activation in dendritic cells and whether it is related to the NGAL induction. For this purpose, BM-DCs from wild-type mice were treated *in vitro* with Aldo for 24 h: Aldo induced a three-fold increase of NGAL mRNA with respect to the control situation, an

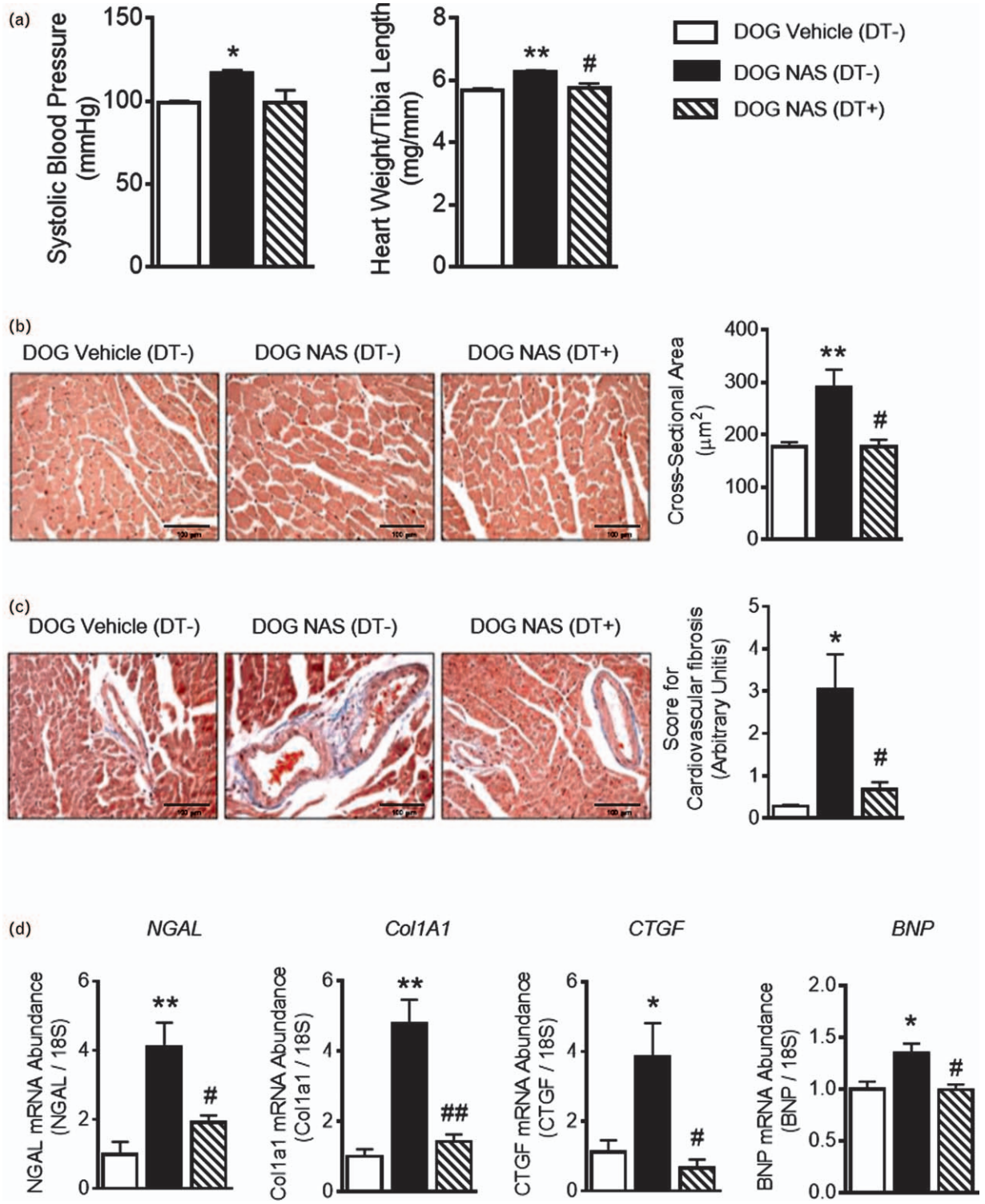

FIGURE 1 Dendritic cells are necessary for the development of mineralocorticoid receptor-dependent cardiac hypertrophy and fibrosis. SBP (left) and heart weight (right) (a), hematoxylin-eosin staining (b), and Masson's trichrome staining (c) in cardiac transverse sections of DOG mice. mRNA abundance performed by quantitative real-time-PCR of neutrophil gelatinase-associated lipocalin, collagen-1, connective tissue growth factor and brain natriuretic peptide in heart of DOG mice (d), after 14 days of treatment. In all cases, 18S was used as housekeeping gene. Mean ± SEM ($n = 5$). $^{*}P < 0.05$ vs. DOG-vehicle (diphtheria toxin−) group, $^{**}P < 0.01$ vs. DOG-vehicle (diphtheria toxin−) group, $^{\#}P < 0.05$ vs. DOG-nephrectomy–aldosterone–salt (diphtheria toxin−) group and $^{\#\#}P < 0.01$ vs. DOG-nephrectomy–aldosterone–salt (diphtheria toxin−) group.

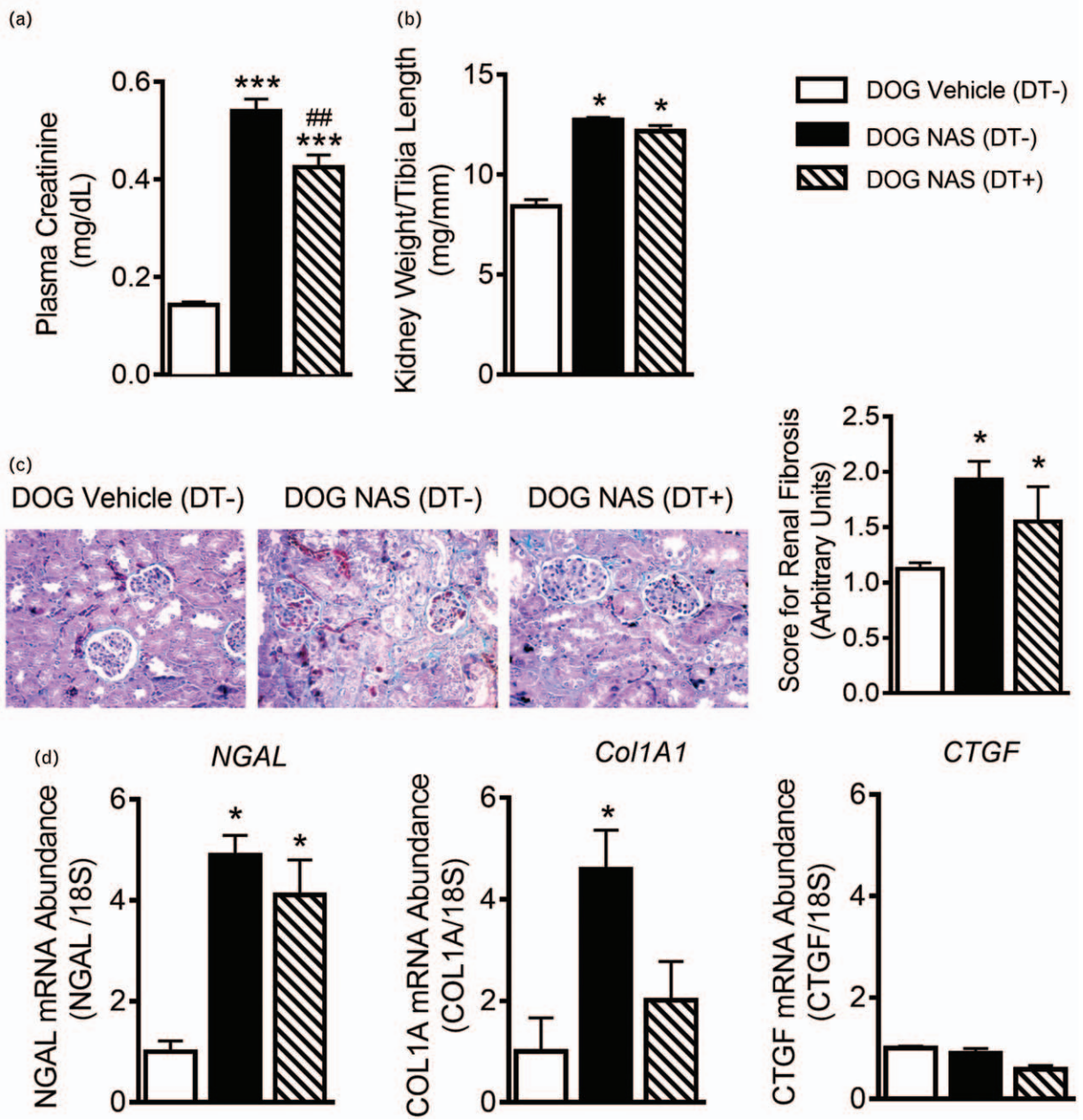

FIGURE 2 The absence of dendritic cells does not prevent renal hypertrophy neither fibrosis Induced by nephrectomy-aldosterone-salt. Plasma creatinine (a), renal weight (b), and Masson's trichrome staining in renal sections of DOG mice (c). mRNA abundance of, neutrophil gelatinase-associated lipocalin, collagen-1, and connective tissue growth factor in kidney of DOG mice (d), after 14-days of treatment. In all cases, 18S was used as housekeeping gene. Mean ± SEM ($n = 5$). $^{*}P < 0.05$ vs. DOG-vehicle (diphtheria toxin−) group, $^{***}P < 0.001$ vs. DOG-vehicle (diphtheria toxin−) group, $^{\#\#}P < 0.01$ vs. DOG-nephrectomy−aldosterone−salt (diphtheria toxin−).

effect fully prevented by mineralocorticoid receptor blockade (Fig. 3b).

Neutrophil gelatinase-associated lipocalin is mandatory for the expression of IL-23 subunits in dendritic cells

As mineralocorticoid receptor activation in dendritic cells promotes Th17 inflammatory response [6], which in turns, has been related to cardiovascular fibrosis in a context of mineralocorticoid excess [7], our rationale was to identify whether NGAL modulates the expression of IL-23 in dendritic cells, a key cytokine secreted by APC required for the induction of Th17-mediated inflammation [25].

BM-DCs were obtained from wild-type and NGAL-knockout mice. NGAL deficiency in dendritic cells did not affect their differentiation as shown by the similar CD11c expression levels in these cells compared with dendritic cells derived from wild-type mice (Fig. 4a). Aldo treatment in wild-type-dendritic cells (24 h) increased NGAL mRNA abundance (Fig. 4b) as well as the mRNA abundance for IL-23, p19 (IL-23p19) and IL-23, p40 subunits, an effect dependent of NGAL expression as it is blunted in NGAL-knock out BM-DCs (Fig. 4c and d, respectively).

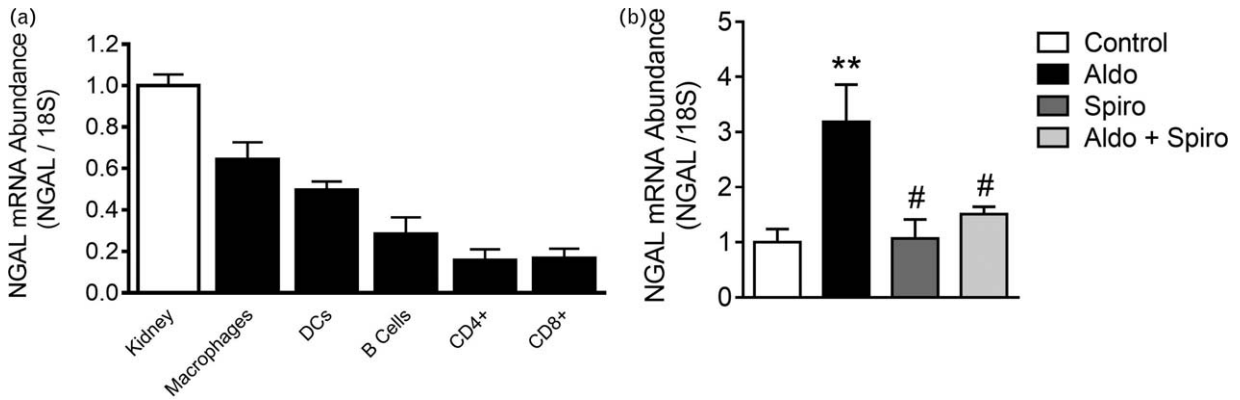

FIGURE 3 Neutrophil gelatinase-associated lipocalin is highly expressed in antigen-presenting cells and is further increased in bone marrow-derived dendritic cells upon mineralocorticoid receptor activation. Neutrophil gelatinase-associated lipocalin mRNA abundance in immune cells after cell sorting from wild-type mice spleen (a). Bone marrow-derived dendritic cells were treated with vehicle (control), aldosterone (100 nmol/l), Spironolactone 5 μmol/l, or aldosterone and Spiro during 24 h for neutrophil gelatinase-associated lipocalin expression analysis (b). Kidney was used as a positive control for neutrophil gelatinase-associated lipocalin expression and 18S was used as housekeeping gene. Mean ± SEM ($n = 3$ for cell-sorting; $n = 6$ for bone marrow-derived dendritic cells).

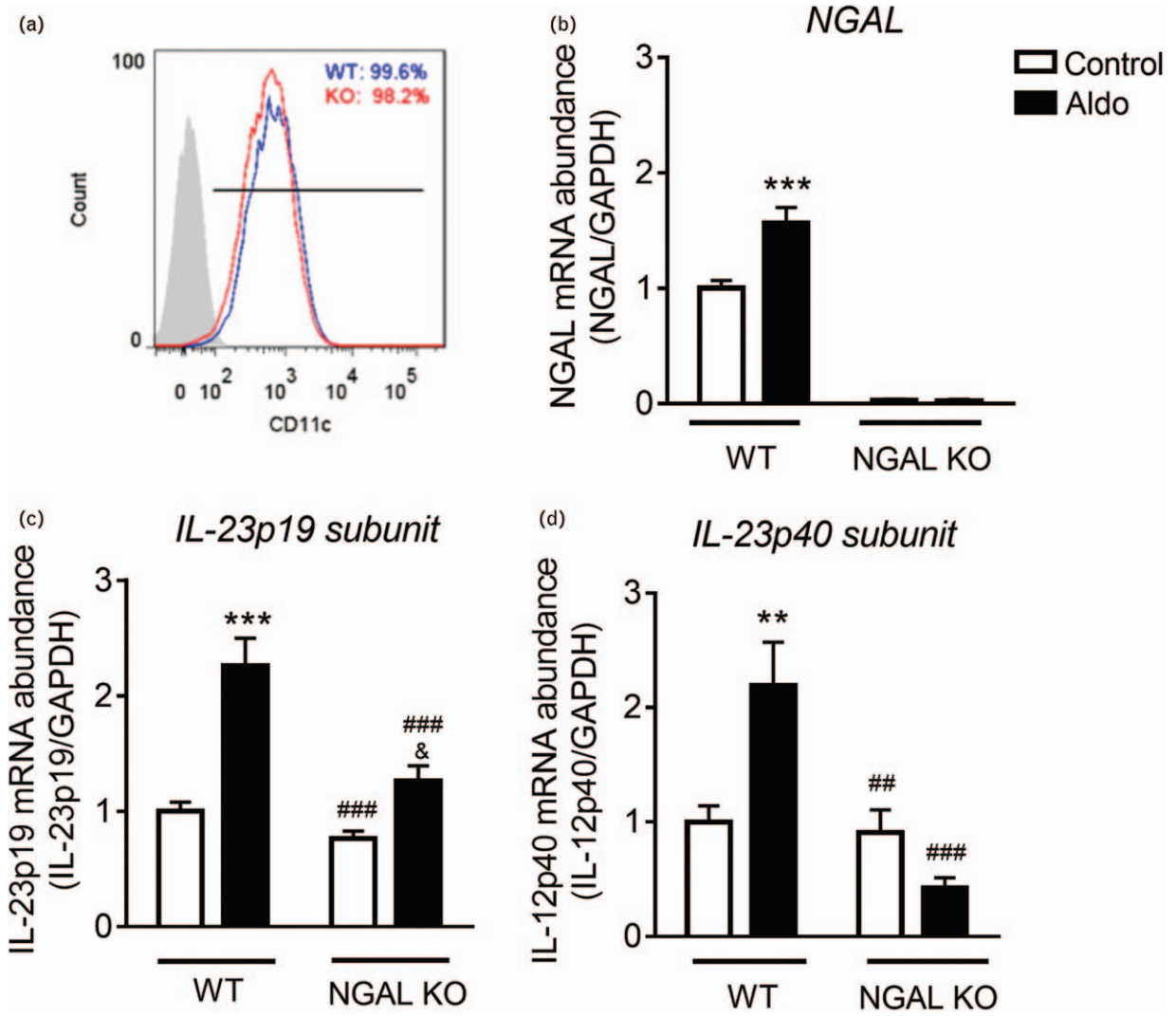

FIGURE 4 Neutrophil gelatinase-associated lipocalin selectively modulates the expression of IL-23 subunits by dendritic cells upon mineralocorticoid receptor activation. Flow cytometry analysis of CD11c expression on bone marrow-derived dendritic cells from wild-type (blue) and neutrophil gelatinase-associated lipocalin-knock out (red). Unstained cells were used as negative control for CD11c expression (gray) (a). mRNA abundance of neutrophil gelatinase-associated lipocalin (b), IL-23, p19 subunit (c), IL-23, p40 subunit (d), transforming growth factor beta 1 (e) and IL-6 (f) was evaluated in wild-type and neutrophil gelatinase-associated lipocalin-knock out bone marrow-derived dendritic cells treated with vehicle (control) or aldosterone (100 nmol/l) during 24 h. Mean ± SEM ($n = 5$), $^{**}P < 0.01$ vs. control wild-type dendritic cells, $^{***}P < 0.001$ < 0.001 vs. control wild-type dendritic cell, $^{\#}P < 0.05$ vs. aldosterone-treated wild-type dendritic cell, $^{\#\#}P < 0.01$ vs. aldosterone-treated wild-type dendritic cell, $^{\#\#\#}P < 0.001$ vs. aldosterone-treated wild-type dendritic cell, $^{\&}P < 0.05$ vs. control neutrophil gelatinase-associated lipocalin-knock out dendritic cell.

Neutrophil gelatinase-associated lipocalin is dispensable for changes in dendritic cells phenotype or T-cell activation after mineralocorticoid receptor activation

Previous studies have shown that RAAS activation induces a proinflammatory phenotype in dendritic cells, characterized by an increase of CD86, a costimulatory molecule, and by significant changes in major histocompatibility complex class I or class II (MHC-I and MHC-II, respectively) [26]. In this context, and according to our results showing the relevance of NGAL to induce IL-23 after mineralocorticoid receptor activation in dendritic cells,

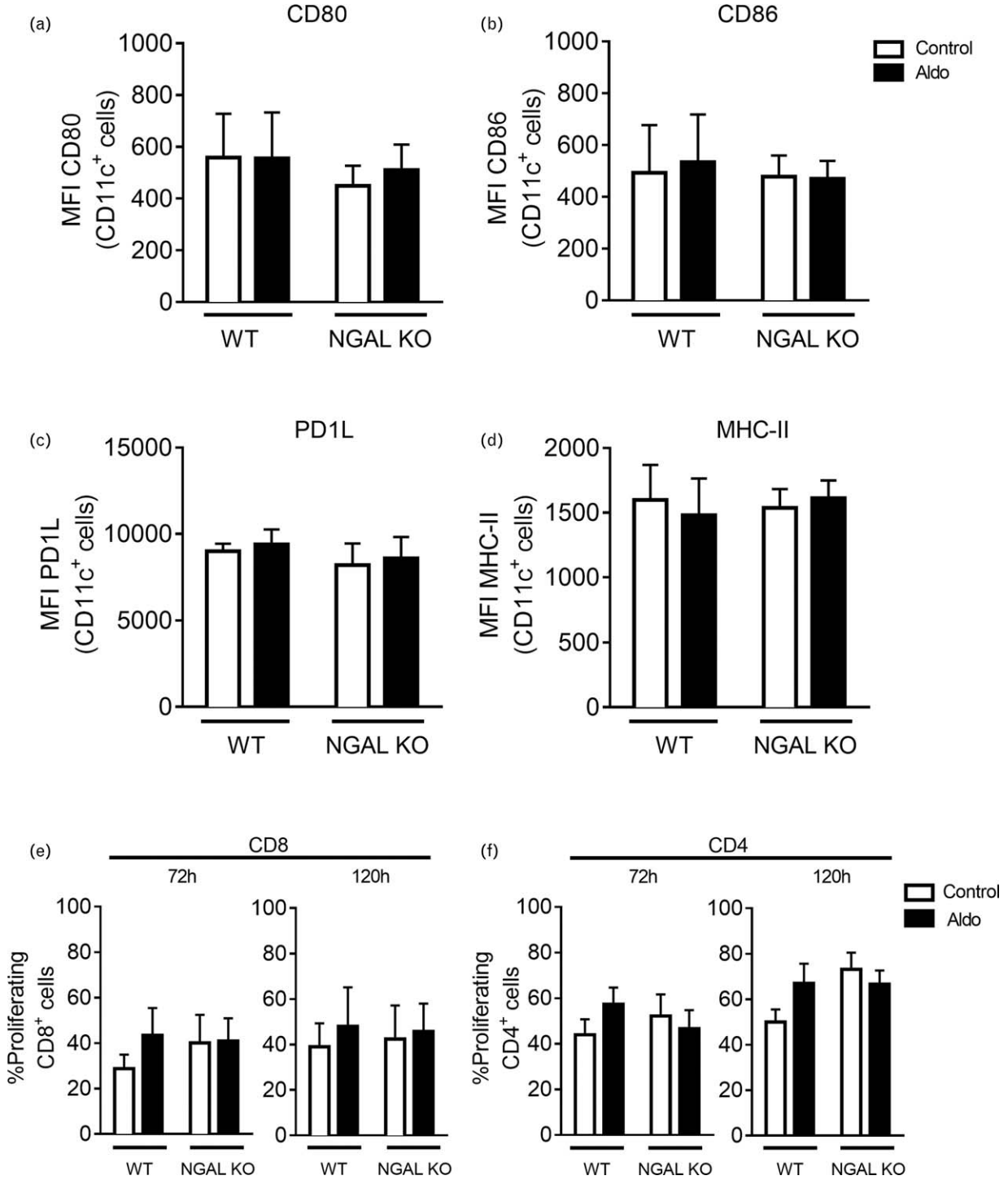

FIGURE 5 Neutrophil gelatinase-associated lipocalin is not mandatory for changes in dendritic cells phenotype, and their capacities to activate T-lymphocytes upon mineralocorticoid receptor activation. Wild-type or neutrophil gelatinase-associated lipocalin-knock out bone marrow-derived dendritic cells were treated with vehicle (control) or aldosterone (100 nmol/l) during 24 h and surface expression of CD80 (a), CD86 (b), programmed death-ligand 1 (c) and major histocompatibility complex-II (d) was measure by flow cytometry. Naïve T cells from OT-I mice (CD8) or OT-II mice (CD4) were cocultured with vehicle or neutrophil gelatinase-associated lipocalin treated-bone marrow-derived dendritic cells and CD8 (e) or CD4 (f) proliferation was observed at indicated times mean ± SEM ($n = 5$).

we evaluated the ability of Aldo to change the phenotype of wild-type and NGAL-knock out dendritic cells. We observed that Aldo treatment do not change expression of costimulator (CD80, CD86) or corepressor (PD-Ll) markers, nor the MHC-II abundance in wild-type or NGAL-knock out dendritic cells compared with the control group (Fig. 5a–d). To evaluate the proliferation of $CD4^+$ and $CD8^+$ T cells after mineralocorticoid receptor activation in wild-type and NGAL-knock out dendritic cells, we performed cocultures with naïve T cells obtained from OT-I mice ($CD8^+$ T cells) and OT-II mice ($CD4^+$ T cells). Aldo treatment did not modify the proliferation rate of $CD8^+$ T cell and $CD4^+$ T cells neither at 72 h nor at 120 h (Fig. 5e and f). The absence of NGAL in dendritic cells was not impacting CD8 or CD4 T cells proliferation after mineralocorticoid receptor activation at both time points (Fig. 5e and f).

As the activation of NGAL receptor, also named 24p3R, has been proposed as a proinflammatory mechanism in organ damage [27–29], we studied the direct effect of exogenous NGAL in dendritic cells. The treatment of wild-type BM-DCs with recombinant murine NGAL during 24 h did not affect their phenotype, assessed by the abundance of surface markers (Fig. S5A, http://links.lww.com/HJH/B72). We did not observe differences between control and NGAL group for CD8 or CD4 T cells proliferation in BM-DCs cocultured with naïve T cells, (Fig. S5B and C, http://links.lww.com/HJH/B72). Finally, we confirmed that treatment of BM-DCs with recombinant NGAL do not modulate polarization to Th1 (IFN-γ and T-box transcription factor) or Th17 (IL-17 and RORγT) profile in CD4 T cells compared with control conditions (Fig. S5D and E, http://links.lww.com/HJH/B72).

DISCUSSION

Dendritic cells have the unique capacity to induce polarization, proliferation, and activation of naïve $CD4^+$ and $CD8^+$ T cells, which in turn, have been closely related with the immune response required for mineralocorticoid receptor-dependent hypertension and target organ damage [6,7,30–32]. We used CD11c.DOG-mice treated with diphtheria toxin to induce-specific dendritic cells depletion in the NAS mouse model [22]. This dendritic cells ablation prevented high blood pressure, cardiac hypertrophy, perivascular fibrosis, and NGAL induction (Fig. 1), indicating that dendritic cells are required to trigger NAS-induced cardiovascular remodeling. Further studies assessing the impact of dendritic cells on myocardial function are needed to elucidate the relevance of dendritic cells in Aldo-dependent heart damage models. Supporting this pivotal role of $CD11c^+$ dendritic cells on cardiac remodeling, Wang *et al.* [33] found that depletion of $CD11c^+$ BM-DCs significantly attenuate cardiac left ventricular (LV) hypertrophy and fibrosis after transverse aortic constriction in mice. However, Anzai *et al.* [34] found that selective depletion of BM-derived $CD11c^+$ dendritic cells during myocardial infarction exacerbated the remodeling of LV, accelerated cardiac dilatation and it reduced LV-fractional shortening, in comparison with control mice. This apparent discrepancy suggests that the participation of dendritic cells in cardiac remodeling may depend on the type of cardiac damage model.

The current study showed that dendritic cells are involved in blood pressure control in mineralocorticoid-dependent hypertension. We recently reported that the $CD11c^+$ population is also responsible for hypertension development in AngII + salt model [5]. Other studies performed by Wenzel *et al.* [35] and Kossmann *et al.* [36] showed that $LysM^+$ cells depletion prevented the onset of high blood pressure in AngII-infused mice, suggesting that $LysM^+CD11b^+CD11c^{high}$ cells could be responsible for hypertension development. Regarding the connection between blood pressure and dendritic cells, the group of Dr Harrison showed that dendritic cells and T-lymphocytes interaction is necessary for hypertension induced by AngII and DOCA [32]. In this interaction, dendritic cells secrete cytokines such as IL-1β, IL-6, and IL-23, activating $CD8^+$ and $CD4^+$-T cells that migrate into the kidney and peripheral vessels. It has also been shown in kidney that Th1 and Th17 lymphocytes generate an increase in sodium (Na^+) reabsorption mediated by specific cytokines produced by these cells. Specifically, Kamat *et al.* [28] showed that mice deficient in IL-17A or IFN-γ exhibit alterations in proximal or distal tubule Na^+ transporters, such as Na^+ hydrogen exchanger-3 and Na^+ chloride cotransporter, leading to enhanced pressure natriuresis and decreased distal Na^+ reabsorption, respectively. We have recently reported that depletion of dendritic cells promotes urinary Na^+ excretion in an AngII/high-salt diet hypertension model [5]. In the present work, we propose that part of the prevention in the increase in blood pressure upon NAS could be mediated by mechanisms that involve increased natriuresis, based on the prevention of renal IFN-γ induction in the NAS CD11c.DOG (diphtheria toxin+) mice (Supplemental data, Fig. S3B, http://links.lww.com/HJH/B72). However, we cannot discard other possible mechanisms related to vasoconstriction for example that directly promotes high blood pressure in the NAS model.

NGAL has been usually considered as a biomarker of kidney injury, which we also confirmed in NAS-treated mice (Fig. 2d). However, dendritic cells depletion did not prevent renal fibrosis. Cumulative evidences suggest that the role of dendritic cells in adaptive immunity is tissue-specific, due to the relationship between tissue-selective subclasses of dendritic cells and the tissue-specific factors that influence the outcome of adaptive-immune responses. Our results suggest that NAS may change the phenotype of dendritic cells to a proinflammatory profile in the kidney, as evidenced by CD86 and MHC-II mRNA increase (Fig. S4C, http://links.lww.com/HJH/B72), without affecting dendritic cells recruitment. Hevia *et al.* [5] demonstrated that AngII+salt induces hypertension and cardiac hypertrophy in CD11c.DOG mice, without increasing dendritic cells recruitment in the target organs they studied. This differential effect between the cardiovascular and renal systems is in accordance with a recent study performed in patients showing that the correlation between NGAL abundance and cardiovascular risk, which was independent of renal dysfunction [37]. A second clinical study revealed that serum NGAL levels in patients with cardiovascular risk are two-fold increase in comparison with control patients, independently their renal function [16]. However, it is needed accumulate more data to understand the effect of

dendritic cells at cardiovascular and renal level in different inflammatory contexts.

Different studies are in accordance with our observations that NGAL is highly expressed in APC in comparison with lymphocytes [8,38,39]. In addition, we determined that NGAL mRNA is increased in wild-type BM-DCs after Aldo treatment, which was prevented by mineralocorticoid receptor antagonism. We have recently reported a positive correlation between NGAL and mineralocorticoid receptor expression in several immune cells, which was particularly evident in dendritic cells and macrophages [20]. The direct modulation of NGAL expression after mineralocorticoid receptor activation has been observed in different cardiovascular cell types, supporting a relevance of the mineralocorticoid receptor/NGAL pathway in cardiac remodeling and blood pressure control. The results presented now include dendritic cells in the modulation of cardiovascular damage through the mineralocorticoid receptor/NGAL axis.

Mineralocorticoids are able to induce Th17-mediated immunity in mice [6] and rats [7] being crucial for tissue damage during mineralocorticoid receptor-dependent hypertension [40]. A critical signal release from dendritic cells to T cells during adaptive inflammatory response is the release of specific cytokines to induce a particular phenotype. In this context, IL-23 is a heterodimeric cytokine composed by p19 and p40 subunits, which are secreted by dendritic cells thereby activating the IL-23 receptor in lymphocytes to induce Th17 polarization/stabilization during chronic inflammation in adaptive immunity [25]. In addition, it has been observed that mineralocorticoid receptor antagonism prevents the increase of IL-23p19 in heart, kidney and white blood cells in DOCA-Salt rats [7]. Here, we showed that Aldo increases both IL-23 subunits in BM-DCs through NGAL expression (Fig. 4). Leopold summarized the NGAL and IL-23/Th17 phenotype interplay in the cardiovascular context, where IL-17A secreted by Th17 may activate NGAL transcription in other cardiovascular cell types, leading to cardiac fibrosis as a consequence [41]. This scenario is particularly relevant considering that NGAL has a direct proinflammatory and profibrotic effect by the induction of IL-6, IL-1β, osteopontin, and Col1A1 in cardiac fibroblast [20]. We now propose that mineralocorticoid receptor activation in cardiac dendritic cells may reinforce the fibrotic phenotype, not only by cardiovascular effects of dendritic cells-released NGAL but also by activating T-lymphocytes to a Th17 phenotype through NGAL-dependent IL-23 secretion.

To deeper analyze the T-cell activation during mineralocorticoid receptor activation and subsequent NGAL induction in dendritic cells, we performed studies of immunological synapse in cocultures [42,43]. Neither BM-DCs maturation phenotype nor dendritic cells capabilities of activating T cells were modified by mineralocorticoid receptor activation in cells obtained from wild-type or NGAL-knockout mice. We also explored whether activation of NGAL receptor (24p3R) in BM-DCs participates in T-cell activation. The treatment of wild-type BM-DCs with recombinant NGAL did not modify their maturation or their capabilities to activate T cells *in vitro*. Significantly, Floderer *et al.* [39] showed that NGAL secreted from BM-DCs can modulate the survival of $CD8^+$ cells *in vitro,* promoting the priming of T cells to proinflammatory Th1 phenotype. Nevertheless, those experiments were performed with a pretreatment of LPS in BM-DCs wild-type and knock out for NGAL, which mimics an infectious state by activating Toll-like receptors; a situation that differs of the sterile inflammation involved in mineralocorticoid-dependent hypertension and cardiovascular fibrosis.

In conclusion, our study demonstrates that dendritic cells are crucial for cardiovascular hypertrophy and fibrosis induced by mineralocorticoids. NGAL abundance is increased in response to mineralocorticoid receptor activation in dendritic cells, where NGAL is required for upregulation of IL-23 after Aldo treatment. The identification of NGAL as a link between mineralocorticoids and IL-23 expression in dendritic cells increases the rationale for understanding the role of dendritic cells and its relevance as a therapeutic target in hypertension and cardiovascular disease. However, the mechanism of IL-23 regulation and consequent effects on T cell by NGAL remains unknown. Further studies are needed to better clarify this mechanism and how is mediating cardiovascular hypertrophy and fibrosis.

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Conflicts of interest

There are no conflicts of interest.

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