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Anakinra reduces blood pressure and renal fibrosis in one kidney/DOCA/salt-induced hypertension

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A B S T R A C T

Objective: To determine whether a clinically-utilised IL-1 receptor antagonist, anakinra, reduces renal inflammation, structural damage and blood pressure (BP) in mice with established hypertension.

Methods: Hypertension was induced in male mice by uninephrectomy, deoxyxorticosterone acetate (2.4 mg/d, s.c.) and replacement of drinking water with saline (1K/DOCA/salt). Control mice received uninephrectomy, a placebo pellet and normal drinking water. 10 days post-surgery, mice commenced treatment with anakinra (75 mg/kg/d, i.p.) or vehicle (0.9% saline, i.p.) for 11 days. Systolic BP was measured by tail cuff while qPCR, immunohistochemistry and flow cytometry were used to measure inflammatory markers, collagen and immune cell infiltration in the kidneys.

Results: By 10 days post-surgery, 1K/DOCA/salt-treated mice displayed elevated systolic BP (148.3 ± 2.4 mmHg) compared to control mice (121.7 ± 2.7 mmHg; n = 18, P = 0.0001). The intervention with anakinra reduced BP in 1K/DOCA/salt-treated mice by ~20 mmHg (n = 16, P < 0.05), but had no effect in controls. In 1K/DOCA/salt-treated mice, anakinra modestly reduced (~30%) renal expression of some (CCL5, CCL2; n = 7–8; P < 0.05) but not all (ICAM-1, IL-6) inflammatory markers, and had no effect on immune cell infiltration (n = 7–8, P > 0.05). Anakinra reduced renal collagen content (n = 6, P < 0.01) but paradoxically appeared to exacerbate the renal and glomerular hypertrophy (n = 8–9, P < 0.001) that accompanied 1K/DOCA/salt-induced hypertension.

Conclusion: Despite its anti-hypertensive and renal anti-fibrotic actions, anakinra had minimal effects on inflammation and leukocyte infiltration in mice with 1K/DOCA/salt-induced hypertension. Future studies will assess whether the anti-hypertensive actions of anakinra are mediated by protective actions in other BP-regulating or salt-handling organs such as the arteries, skin and brain.

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; BP, blood pressure; CARD, caspase activation and recruitment domain; CCL2, chemokine ligand 2; CCL5, chemokine ligand 5; cDNA, complementary DNA; DAMP, danger-associated molecular pattern; DOCA, deoxyxorticosterone acetate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBP, hereditary hypertension; HSP, heat shock protein; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin 1β; IL-1RA, interleukin 1 receptor antagonist; IL-1βR, interleukin 1β; IL-6, interleukin 6; mmHg, millimetres of mercury; MW, molecular weight; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLR, nod-like receptor; NLRP, nod-like receptor with caspase-recruitment domain; NLRP1, nod-like receptor with pyrin domain; NLRP3, nod-like receptor with pyrin domain containing 3; PAMP, pathogen-associated molecular pattern; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PRR, pattern recognition receptor; RNA, ribonucleic acid; SFP, specific pathogen free; TLR, toll-like receptor; VCAM-1, vascular cell adhesion molecule 1.

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1. Introduction

Hypertension is now recognised as a low-grade inflammatory condition with growing evidence to suggest that the cytokine, IL-1β, plays an important role. Previous studies have shown that hypertensive patients have increased circulating levels of IL-1β, and furthermore that levels of IL-1β are positively correlated with increasing blood pressure (BP) [1,2]. In a prospective study, it was found that levels of IL-1β were significantly higher in those normotensive individuals who went on to develop hypertension during the 6.5 year follow up [2]. This latter finding showing that augmented levels of IL-1β precede rises in chronic BP is suggestive of a causal role for the cytokine in the pathogenesis of hypertension.

Indeed, a causative role for IL-1β is also supported by recent preclinical findings. IL-1β is formed from its inactive precursor, pro-IL-1β, by the actions of a class of innate signalling complexes known as inflammasomes [3]. Inflammasomes are multitimeric protein complexes comprising of one of four pattern recognition receptors (PRR) (NLRP1, NLRP3, AIM2, NLRC4) responsible for the detection of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) [3]: an adaptor protein, ASC; and pro-caspase-1, which is ultimately responsible for cleaving pro-IL-1β into its active form. Studies by our group and others have shown that various forms of hypertension in mice are associated with increased expression of inflammasome subunits and IL-1β in the kidneys. Moreover, genetic deficiency of ASC or NLRP3, or treatment with an IL-1β neutralising antibody, afforded mice protection from hypertension, renal inflammation and fibrosis [4,5]. Collectively, the above findings highlight the inflammasome/IL-1β signalling pathway as a potential target for new therapies to treat hypertension.

Anakinra is a recombinant form of the endogenous human interleukin-1 receptor antagonist (IL-1Ra) that prevents IL-1β signalling by competitively binding to the IL-1 receptor (IL-1R) [6]. Anakinra is approved clinically for the treatment of chronic inflammatory diseases such as rheumatoid arthritis and neonatal-onset multisystem inflammatory disease. However, whether anakinra can also be used as a treatment for hypertension has not been examined. Therefore, in the present study we evaluated the effects of an intervention with anakinra on BP and markers of renal inflammation and damage in one kidney/deoxycorticosterone acetate/salt-dependent hypertension in mice.

2. Methods

2.1. Animals

178 male C57BL/6J mice, of age 10–12 weeks and weighing 25–30g, were used in this study. Mice were obtained from the Monash Animal Research Platform (MARP; Monash University, Australia) or the Animal Resources Centre (Perth, Australia). Mice were housed under specific pathogen free conditions, on a 12 h light-dark cycle and provided with ad libitum access to normal chow and drinking water. All procedures were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and were approved by the MARP Animal Ethics Committee (Project number: MARP/2013/043).

2.2. Induction of Hypertension

Mice were placed under general anaesthesia by isoflurane inhalation (2.2–2.6% isoflurane in 100% O₂). While under anaesthesia, mice were monitored for hind-paw withdrawal, blink reflexes and respiratory rate. Hypertension was induced by removal of the left kidney, implantation of a deoxycorticosterone acetate (DOCA) pellet (2.4 mg/d, 21 d; Innovative Research of America, USA) into a small subcutaneous pouch in the left scapular region, and replacement of the drinking water with 0.9% saline (1K/DOCA/salt) [7]. Normotensive mice were also uninephrectomised but received a placebo pellet and were maintained on normal drinking water (1K/placebo).

2.3. Anakinra intervention protocol

10 days after surgery to induce hypertension, some mice were further treated with either a low dose of anakinra (25 mg/kg/d; Amgen, USA), a higher dose of anakinra (75 mg/kg/d) [8] or vehicle (0.9% saline, 100 μl) for the remaining 11 days of the experiment via daily intraperitoneal (i.p.) injections. A cohort of normotensive mice was also treated with anakinra or vehicle from 10 to 21 days post-surgery. These doses of anakinra were chosen as they fall within the range of doses that have previously been shown to be efficacious in other models of disease in mice [9,10]. Importantly, we saw no signs of any adverse side-effects with these doses of anakinra (e.g. weight loss, general appearance and behaviour, mortality rate, etc.).

2.4. Blood pressure measurements

Systolic BP was measured via tail cuff plethysmography using a multi-channel BP analysis system (MC4000; Hatteras Instruments, USA). BP was recorded for 30–40 measurement cycles daily for at least 3 days prior to surgery to acclimatise mice to the procedure. BP was then measured just prior to surgery (day 0) and again on days 3, 7, 10, 14, 17, and 21.

2.5. Renal messenger RNA (mRNA) expression of inflammatory markers

Twenty-one days after surgery, mice were killed by isoflurane overdose (Baxter Healthcare, Australia) and perfused through the left ventricle with 0.2% clexane (400 IU, Sanofi Aventis, Australia) in RNase-free phosphate-buffered saline (PBS). The right kidney was excised and cut transversely with a scalpel blade. Half of the kidney was set aside for immunohistochemistry or flow cytometry, while the other half was further divided into two equal portions, each of which were placed in a microcentrifuge tube and snap frozen in liquid nitrogen. RNA was extracted from the same portion of these frozen samples using a commercially available RNA extraction kit (RNeasy Mini Kit; Qiagen, USA). The concentration and purity of the RNA was determined by measuring absorbance at 230, 260, and 280 nm with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). Samples were considered to be sufficiently free of protein and phenol contamination and suitable for PCR analysis when the 260/280 and 260/230 ratios were ≥2.

RNA was reverse transcribed to cDNA using a commercially available kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA). cDNA was then used as a template for real-time PCR to measure mRNA expression of NLRP3, ASC, pro-caspase-1, pro-IL-1β, pro-IL-18, IL-1R, IL-18Ra/P, CCL5, CCL2, ICAM-1, VCAM-1, IL-6, collagen type 1 alpha 1 (COL1A1), collagen type 3 alpha 1 (COL3A1), collagen type 4 alpha 1 (COL4A1), collagen type 5 alpha 1 (COL5A1) and the house keeping gene, GAPDH (Taqman Gene Expression Assays, Applied Biosystems, USA). Real-time PCR was then performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Australia) and the following thermocycling parameters: an initial step of 50 °C for 2 min; 10 min at 95 °C; 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Fluorescence was measured at the end of each cycle. Fold-changes in mRNA expression relative to the 1K/placebo + saline samples were determined using the comparative CT method [11].
2.6. Flow cytometric analysis of leukocyte infiltration into the kidney

Single cell suspensions were prepared from half kidney samples. Briefly, kidneys were minced with scissors and digested by incubation for 30 mins at 37 °C in PBS containing collagenase type XI (125 U/ml), collagenase type I-S (460 U/ml) and hyaluronidase (60 U/ml) (Sigma-Aldrich, USA). Samples were passed through a 70 μm filter before being subjected to a Percoll™ gradient spin. The layer containing mononuclear cells was transferred to a fresh tube and stained with an antibody cocktail consisting of anti-mouse CD45 (APC-Cy7; BioLegend, USA), CD11b (PacB; eBioscience, USA), Ly6G (PE-Cy7; BioLegend, USA), CD3 (V500; BD Bioscience, USA), CD4 (FITC; eBioscience, USA), CD8 (BV605; BioLegend, USA), F4/80 (APC; eBioscience, USA), and CD206 (PE; BioLegend, USA) diluted in PBS containing 0.5% bovine serum albumin. Samples were then analysed by flow cytometry using a BD LSR Fortessa™ (BD Bioscience, USA) and FlowJo Software (version 10.1, Tree Star Inc, USA). Cell numbers were normalised using CountBright counting beads (Invitrogen, USA) and expressed as total cells per kidney.

2.7. Picosirius red staining for the detection of renal interstitial collagen and glomerular cross sectional area (GSA)

Kidney halves (composed of the renal cortex and medulla) were fixed in 10% formalin, embedded in paraffin and sectioned (5 μm). Sections were then deparaffinised, rehydrated and stained with a 0.1% Picosirius red solution. Imaging was performed using a bright-field microscope at ×20 magnification. To determine collagen content, the percentage of area stained by Picosirius red in six randomly selected fields-of-view was quantified by a blinded investigator using Imagej software (National Institutes of Health, USA). To determine glomerular cross sectional area (GSA), the glomerular area was traced and analysed by inbuilt computerised morphometry (DP-21), based on pixel size and magnification. For the purpose of analysis, glomeruli were defined as the matrix, cells, and space within the circumference of the Bowman’s capsule.

2.8. Multiplex immunoassay for quantification of antibody levels in plasma

Blood was collected from the inferior vena cava in ethylene-diaminetetraacetic acid (EDTA)-coated tubes and centrifuged to separate plasma. The concentration of antibody isotypes (IgG1, IgG2a, IgG2b, and IgG3) in plasma was quantified using a ProcartaPlex Mouse Antibody Isotyping Panel (eBioscience, USA). The antibody assay was run on a Bio-Plex™ MAGPIX™ Multi-Plex Reader (BioRad, USA). Data were analysed using ProcartaPlex Analyst (eBioscience, USA).

2.9. Enzyme-linked immunosorbent assay (ELISA) for detection of IL-18 in plasma

An ELISA was performed to determine levels of active IL-18 released into the circulation of 1K/DOCA/salt-treated mice (Medical Biological Laboratories, Japan). The assay was performed according to manufacturer’s instructions in microtiter plates coated with antibodies specific for IL-18. The amount of IL-18 in plasma samples was estimated by calibrating the optical density (OD) values of the samples with the OD values of serially diluted recombinant protein standard.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Systolic BP was analysed by two-way repeated measures ANOVA followed by Bonferroni post-hoc test. All other data were analysed using either Student’s unpaired t-test or two-way ANOVA followed by Newman-Keuls post hoc test. P < 0.05 was considered to be statistically significant. Note: post hoc tests were only performed where the f-ratio of the ANOVA highlighted a significant difference (P < 0.05). Data were graphed and analysed using GraphPad Prism Software v6.04.

3. Results

3.1. Anakinra reduces systolic BP in 1K/DOCA/salt-induced hypertension

1K/DOCA/salt treatment in mice caused an increase in systolic BP that appeared to plateau after 10 d to a level that was approximately 30 mmHg higher than baseline BP (Fig. 1A). Subsequent treatment of hypertensive mice with either the vehicle for anakinra (saline) or with a low dose of the drug (25 mg/kg/d) for the remaining 11 d of the experiment had no apparent effect on BP (Fig. 1A). By contrast, the higher dose of anakinra (75 mg/kg/d) reduced BP by ~15–20 mmHg within 4 d and this effect was largely maintained until the end of the treatment period (Fig. 1A). BP in control mice that underwent 1K/placebo treatment did not deviate from baseline over the first 7–10 d. Moreover, treatment of 1K/placebo mice with either vehicle, low dose anakinra or high dose anakinra, had no further effect on BP (Fig. 1B). These observations suggest that the high dose of anakinra (75 mg/kg/d) is effective at selectively reducing BP in hypertensive but not normotensive mice. Thus, in all subsequent experiments, the effects of only this higher dose of anakinra were evaluated.

3.2. Anakinra reduces renal interstitial collagen deposition in 1K/DOCA/salt-treated mice

Hypertension is associated with renal interstitial fibrosis [12]. Therefore, we determined whether the anti-hypertensive effect of anakinra was accompanied by a reduction in renal collagen levels. Picosirius red staining revealed approximately 8-fold higher levels of interstitial collagen in kidneys of mice that were made hypertensive by 1K/DOCA/salt and subsequently treated with vehicle, than in normotensive mice treated with vehicle (Fig. 2A and B). Consistent with its effects on BP, anakinra inhibited collagen deposition in the kidneys of 1K/DOCA/salt-treated mice, but had no effect in the normotensive animals (Fig. 2A and B).

To determine whether the effects of anakinra on collagen deposition in hypertensive 1K/DOCA/salt-treated mice were mediated at a transcriptional level, we measured mRNA expression of four of the major collagen subtypes, collagen I, III, IV, and V. The expression of each of these collagen subtypes was markedly enhanced in kidneys from 1K/DOCA/salt-treated mice compared to normotensive 1K/placebo-treated animals (Fig. 2C–F). However, unlike its effects on protein levels of interstitial collagen, anakinra did not reduce mRNA levels of any collagen subtypes in either 1K/DOCA/salt- or 1K/placebo-treated mice (Fig. 2C–F).

3.3. Effect of anakinra on markers of renal inflammation and damage

Collagen deposition often occurs in response to inflammation and injury and thus we measured the effects of 1K/DOCA/salt and anakinra treatments on markers of renal inflammation and injury in mice. As per our previous report [4], 1K/DOCA/salt-induced hypertension in mice was associated with elevated renal expression levels of inflammasome subunits including NLRP3, ASC, and pro-caspase-1 (Fig. 3A–C). Also consistent with our previous finding, mRNA expression of pro-IL-1β was elevated in the kidneys of 1K/DOCA/salt-treated mice (Fig. 3D). Intervention with anakinra
Fig. 1. Effect of anakinra on systolic BP in 1K/DOCA/salt (A) and 1K/placebo-treated (B) mice. Blood pressure was measured via tail-cuff plethysmography. 10 days after surgery, mice were further treated with anakinra (75 or 25 mg/kg/d, i.p.) or vehicle (0.9% saline). Data are expressed as mean ± SEM (n = 10–18 per group). ‘***P < 0.0001 for two-way repeated measures ANOVA; #P < 0.05, ###P < 0.001 for Bonferroni multiple comparisons test.

had no effect on expression levels of any of the above inflammasome subunits/cytokines in either 1K/DOCA/salt-treated mice or in normotensive 1K/placebo mice (Fig. 3) suggesting that the drug is likely to be acting downstream of inflammasome priming.

Real-time PCR also revealed that several markers of inflammation and injury previously shown to lie downstream of inflammasome activity/IL-1β were upregulated in the kidneys of hypertensive 1K/DOCA/salt-treated mice relative to normotensive 1K/placebo-treated mice including the chemokines CCL5 and CCL2 (Fig. 4A–B); adhesion molecules ICAM-1 and VCAM-1 (Fig. 4C–D); and the pro-inflammatory cytokine IL-6 (Fig. 4E). Anakinra attenuated 1K/DOCA/salt-induced increases in expression of CCL5 and CCL2 by 25–40% (Fig. 4A–B) and appeared to have a similar inhibitory impact on VCAM-1 expression. However, for this latter observation, the effect failed to reach statistical significance (Fig. 4D). Anakinra intervention also had little to no effect on ICAM-1 or IL-6 expression in the kidneys of 1K/DOCA/salt-treated mice nor did it reduce expression levels of any of the above inflammatory markers in normotensive mice (Fig. 4A–E).

Chemokines play a crucial role in leukocyte trafficking into the kidneys and vascular wall during hypertension [7,13,14]. Thus, given that anakinra reduced renal expression of CCL5 and CCL2 in 1K/DOCA/salt-treated mice, we performed flow cytometric analysis to determine if the drug similarly reduced leukocyte numbers in the kidneys. Relative to normotensive mice, 1K/DOCA/salt-induced hypertension was associated with elevated numbers of total leukocytes in the kidneys (Fig. 5A). Further analysis of the immune cell subsets that contributed to this revealed expansions in the populations of CD4+ T cells, CD8+ T cells and CD206+ (“M2”) macrophages, but no changes in CD206- (“M1”) macrophages or neutrophils (Fig. 5B–F). For each of these cell types, and irrespective of whether the mice were hypertensive (1K/DOCA/salt) or normotensive (1K/placebo), anakinra had no effect on cell number (Fig. 5A–F).

3.4. Effect of anakinra on renal hypertrophy

A previous report suggested that chronic treatment of rats with anakinra was associated with hypertrophy of the kidneys [15]. In the present study we showed that while 11 d of anakinra treatment had no effect on the kidney weight (normalised to body weight) in the normotensive animals, it exacerbated renal hypertrophy in 1K/DOCA/salt-hypertensive animals (Fig. 6A). These effects of anakinra were also reflected at the glomerular level with anakinra causing an increase in the average size of glomeruli within the kidneys of 1K/DOCA/salt-treated mice (Fig. 6B).

4. Discussion

The major finding from this study is that the clinically-used recombinant human IL-1Ra, anakinra, was effective at reversing BP and reducing renal fibrosis in mice with established 1K/DOCA/salt-dependent hypertension. This effect of anakinra occurred despite the drug appearing to have only modest effects on renal inflammation and even exacerbating renal hypertrophy.

Previous studies using a variety of experimental models (1K/DOCA/salt, angiotensin II infusion, renal artery clipping) have demonstrated that inhibition of inflammasome activation or neutralisation of IL-1β prior to the induction of hypertension is effective at preventing subsequent increases in BP [4,5]. In the
present study we demonstrated that an intervention to inhibit IL-1 signalling, initiated after 1K/DOCA/salt-dependent hypertension had become established (i.e. 10 days post-surgery), caused a rapid, albeit partial reversal of BP. Indeed, the magnitude of the effect (15–20 mmHg) is significant when one considers that in hypertensive humans, every 10 mmHg reduction in systolic BP equates to a >20% reduction in risk of coronary heart disease and a >40% reduction in risk of stroke [16]. Importantly the treatment protocol used herein more closely resembles how the condition is managed in the clinic (i.e. treatment is only initiated once hypertension is established and diagnosed). Hence, our findings lend further support to
the concept that the inflammasome/IL-1 signalling pathway is a promising target for novel anti-hypertensive therapies.

Previous studies highlight the kidneys as a major site of inflammasome activation and suggest that this is an important driver of renal inflammation, which in turn leads to interstitial fibrosis and renal dysfunction [4,5,12]. Inflammation and fibrosis in the kidneys are thought to disrupt the pressure-natriuresis relationship, leading to Na⁺/H₂O retention and increased arterial BP [12,17–20], thus providing an explanation of how inflammasome activation may contribute to hypertension. Indeed, in the present study we provided evidence for inflammasome priming as well as upregulation of IL-6 which is purported to be regulated downstream of IL-1R1 signalling [21,22] and several other markers of inflammation in the kidneys of 1K/DOCA/salt-treated mice. These findings are consistent with our previous report where, in addition to demonstrating increased mRNA expression of inflammasome subunits, pro-IL-1β and IL-6, we provided evidence for inflammasome/caspase-1 activation and protection against 1K/DOCA/salt-dependent hypertension and renal inflammation in ASC⁻/⁻ mice [4]. However, in the present study anakinra had little to no effect on measures of renal inflammation, despite its robust anti-hypertensive actions. A potential explanation for the differential effect of anakinra and ASC-deficiency on renal inflammation is that the former intervention will only target IL-1R1 signalling, whereas the latter is likely to inhibit both IL-1β and IL-18-dependent pathways [3]. This may indicate that the IL-18 system is more important than IL-1β for promoting renal inflammation, at least in the context of 1K/DOCA/salt-dependent hypertension. Although we have not yet tested for a possible anti-inflammatory effect in the kidneys of selective inhibition of IL-18 signalling, we do have preliminary evidence to suggest that this arm of the IL-1 family is upregulated in 1K/DOCA/salt-dependent hypertension. Specifically, we have shown that although renal mRNA and circulating levels of IL-18 itself remain unchanged, expression of both the IL-18 receptor (IL-18R) and the IL-18R accessory protein (IL-18RαCp) are markedly upregulated in the kidneys of mice with 1K/DOCA/salt-dependent hypertension (Supplementary Fig. S1).

The previous discussion implies that the BP-lowering effects of anakinra occurred independently of any anti-inflammatory actions in the kidneys. By extension, this suggests that the site of action of anakinra is possibly another BP-regulating organ/tissue. Previous studies have established the important association between vascular inflammation, endothelial dysfunction and hypertension, suggesting that the blood vessels may be one such site. For example, experimentally-induced hypertension in mice is associated with increased expression of inflammatory cytokines in the arterial wall, including IL-1β and IL-6, which is accompanied by infiltration and activation of leukocytes such as T cells, B cells and macrophages [7,23,24]. Inhibition of these inflammatory factors and/or cells using neutralising antibodies, chemokine receptor antagonists or knockout mouse models reduces endothelial dysfunction and BP in hypertensive animals [7,23,24]. While the mechanisms linking inflammation to impaired vasorelaxation in these models have not been fully elucidated, there is some evidence that IL-1β could directly influence these parameters. For example, three independent studies showed that large and resistance-like arteries from rats displayed impaired endothelium-dependent relaxations to (acetylcholine) ACh and augmented contractions to phenylephrine following exposure to IL-1β ex vivo [25–27]. Furthermore, IL-1β-treated vessels generated more superoxide than controls and treatment of the vessels with superoxide dismutase partially reversed the impaired responses to ACh [26] implicating a role for oxidative stress. Based on these previous observations, in pilot studies we investigated the effects of the
1K/DOCA/salt- and anakinra- treatments on endothelial function in second order mesenteric resistance-like arteries (Supplementary Fig. S3). Surprisingly, we neither saw any evidence of endothelial dysfunction in the (saline-treated) 1K/DOCA/salt-treated mice, nor did anakinra appear to augment endothelium-dependent vasorelaxation responses in these animals. Hence, these findings suggest that impaired vasodilator function has only a minor (if any) contribution to chronic pressor effects to 1K/DOCA/salt and, by extension that the BP-lowering actions of anakinra observed here were unlikely to be due restoration/improvement of endothelial function.

Of course, it is also possible that anakinra did reduce renal inflammation in the early stages of treatment. However, by the time of assessment these anti-inflammatory effects had already waned. In such a scenario, it is conceivable that there might be a time lag between reversal of the anti-inflammatory action of anakinra compared to that of downstream processes such as collagen deposition and elevated BP, such that these latter parameters remained attenuated at the end of the 10-day anakinra treatment period. As to potential reasons why the IL-1R-inhibiting effects of anakinra may have been transient, it is worth noting that as a recombinant human protein, anakinra has the potential to induce an immune response when chronically administered to other species. Indeed, we have evidence that hypertensive mice administered with anakinra have higher plasma IgG levels than those animals administered with vehicle (Supplementary Fig. S2), and it is possible that such antibodies could be acting to neutralise the effects of the protein. Regardless, the previous discussion highlights an important limitation of the current study (i.e. inflammation was only assessed at one time-point) and indicates that further analysis of the time-course and long-term actions of anakinra on renal inflammation, fibrosis and BP are warranted. Interestingly, the human monoclonal anti-IL-1β antibody, Canikumab, is currently under investigation for the prevention of recurring cardiovascular events. As this does not involve cross-species protein administration, the problems associated with generation of auto-antibodies to the treatment encountered in the current study will not be applicable. Therefore, it will be interesting to observe any reduction in blood pressure with Canikumab treatment.

![Figure 4](image_url)
Fig. 5. Effect of anakinra on leukocyte infiltration; Total leukocytes (A), T helper cells (B), cytotoxic T cells (C), “M1” macrophages (D), “M2” macrophages (E) and neutrophils (F) in the kidneys of 1K/DOCA/salt and 1K/placebo-treated mice. Leukocyte infiltration was quantified by flow cytometric analysis. Data are expressed as mean ± SEM (n = 11–17 per group). *P < 0.05, ***P < 0.001, ****P < 0.0001 for two-way ANOVA followed by Newman-Keuls multiple comparisons test.

Finally, we observed that anakinra exacerbated renal hypertrophy in 1K/DOCA/salt-treated mice, a finding that is consistent with an earlier study where it was reported that chronic treatment of rats with a high dose of anakinra (200 mg/kg/d) for 6 months similarly induced an increase in kidney weight [15]. While renal hypertrophy is often suggestive of renal injury [28], previous studies demonstrating that anakinra limits renal damage and dysfunction in several disease and injury settings including heatstroke in rabbits [29] and endotoxin- or antibody-mediated nephritis in rodents [30,31], tends to argue against such an effect. Furthermore, our observation that anakinra reduced picrosirius red staining in kidneys of hypertensive mice is also indicative of a protective rather than detrimental action of the drug. Picrosirius red is established to be a valid tool for the detection of collagen networks in tis-
sues [32], and thus the reduction in picrosirius red staining in kidneys from hypertensive mice treated with anakinra is consistent with a protective effect against 1K/DOCA/salt-induced renal fibrosis. Picrosirius red is not suitable, however, for discerning between different subtypes of collagen [32]. Thus, to gain some insight into which collagen subtypes were altered by anakinra treatment, and the mechanisms involved, we measured expression levels of the genes encoding four of the most common types of fibrillar collagens – I, III, IV and V – by real-time PCR. Although expression levels of all of these genes were higher in the 1K/DOCA/salt- versus 1K/placebo-treated mice, none of them appeared to be affected by further treatment with anakinra. There are at least two potential explanations for the apparent discrepancies between these observations and our finding of reduced picrosirius red staining. First, it is plausible that the reduced amounts of interstitial collagen in anakinra-treated animals was the result of enhanced breakdown, rather than decreased production of the protein. Such a process could result from upregulation of matrix metalloproteinases (MMPs) [33], and hence in future studies it may be worthwhile measuring the impact of anakinra treatment on levels of MMPs in the kidneys of 1K/DOCA/salt-treated mice. Alternatively, other classes of collagen(s) may have contributed to the renal interstitial fibrosis observed in this model e.g. Type VI; [34], and it was these classes that were most affected by anakinra treatment. Hence, further characterisation of the extracellular matrix composition of the kidneys via real-time PCR and/or immunostaining is warranted.

In conclusion, we have demonstrated that anakinra – a drug that is already used clinically in the treatment of autoimmune and inflammatory diseases such as rheumatoid arthritis and gout – is effective at reducing BP and renal collagen deposition in mice with established hypertension. While questions remain around the precise mechanisms involved in the anti-hypertensive actions of anakinra, our results are further evidence of the immune basis of hypertension and provide proof-of-concept that interventions targeting immune dysregulation, hold promise as future therapies for the condition.

5. Conflicts of interest

The authors have no conflicts of interest to declare.

6. Authorship contribution

YHL, SMK, GRD, BKK, CSS, EL, AM, CGS and JC-D wrote the paper.

7. Funding disclosure

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Appendix A.

Previous presentations


Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.12.015.

References


