The role of inflammasomes, IL-1β, IL-18, and nitric oxide in inflammation and hypertension

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Table of Contents

**Chapter 3: Anakinra reduces blood pressure and renal fibrosis in one
kidney/DOCA/salt-induced hypertension 83**

Abstract

Over the past decade it has emerged that hypertension is associated with the accumulation of immune cells in key blood pressure-regulating organs such as the blood vessels and kidneys. Through the release of cytokines and other pro-inflammatory factors, these cells contribute to the establishment of the chronic inflammatory state which impairs renal and vascular function and exacerbates hypertension. The NLRP3 inflammasome is a multimeric protein complex that regulates production of the pro-inflammatory cytokines, IL-1β and IL-18. It is implicated in several 'classical' inflammatory diseases including gout, rheumatoid arthritis and atherosclerosis, and recent findings from our group have shown that the NLRP3 inflammasome is also likely to be a crucial mediator of the chronic inflammation that develops in the kidney during hypertension.

Specifically, we showed that mice genetically deficient in a key subunit of the NLRP3 inflammasome, namely the adaptor protein ASC, are protected against renal inflammation, fibrosis and the development of high blood pressure following chronic treatment with either deoxycorticosterone acetate/salt, or angiotensin II. However, it remains to be determined: (a) which of the inflammasome-derived cytokines – IL-1β, or IL-18 – is responsible for promoting renal inflammation and hypertension; and (b) whether inflammasome activity can be targeted pharmacologically in a more clinically-relevant intervention scenario to treat hypertension once the disease is already established. This project aims to address these knowledge gaps.

In Chapter 3, we utilised a recombinant human IL-1 receptor antagonist, anakinra, to investigate the role of IL-1β in the development of 1K/DOCA/salt-induced hypertension in mice. Although anakinra was effective at reducing BP and renal collagen deposition in hypertensive mice, it had negligible effects on renal inflammation and even exacerbated renal hypertrophy, possibly indicating that it promoted further damage to the kidneys. So, while the findings of this study implied that IL-1β does indeed play some role in the

regulation of blood pressure in hypertensive mice, this would appear to occur largely independent of any major pro-inflammatory actions in the kidneys.

In Chapter 4, we studied mice that were genetically deficient in IL-18 (IL-18$^{-/-}$) to test whether IL-18 might be playing a more prominent role in renal inflammation during 1K/DOCA/salt-induced hypertension. We found that compared to wild-types, IL-18$^{-/-}$ mice were profoundly protected from 1K/DOCA/salt-induced renal inflammation, as assessed by immune cell infiltration, T cell activation and expression of inflammatory markers in the kidneys. IL-18$^{-/-}$ mice also displayed less renal interstitial collagen deposition and a blunted hypertensive response to 1K/DOCA/salt. Hence, IL-18 appears to be a major contributor to renal inflammation and a promising target for future reno-protective and anti-hypertensive therapies.

In Chapter 5 we investigated whether the previously-described anti-inflammatory actions of nitric (NO) donors might involve inhibition of NLRP3 inflammasome-dependent signalling. Using cultured mouse and human macrophage cell lines, we showed that the NO donors, SIN-1 and spermine-NONOate were powerful inhibitors of LPS-induced upregulation of NLRP3 and IL-1β mRNA expression. The NO donors also inhibited inflammasome upregulation in response to other stimuli including CpG (TLR9 agonist) and imiquimod (TLR7 agonist). The inhibitory effect of SPER-NO on inflammasome expression was partially prevented by a NO scavenger, hydroxocobalamin, but was not affected by a guanylyl cyclase (sGC) inhibitor, ODQ. Hence, while further work is required to elucidate the precise mechanism by which NO donors inhibit inflammasome expression, this study nonetheless highlights the potential of NO donors as future anti-inflammatory/anti-hypertensive agents.

In Chapter 6, we sought to determine whether a recently-identified small-molecule inhibitor of NLRP3 inflammasome activity, MCC950, could reduce blood pressure and reverse renal inflammation in mice with established 1K/DOCA/salt-induced hypertension. An intervention

with MCC950 initiated 10 days after induction of 1K/DOCA/salt hypertension was highly effective at reducing expression of several inflammatory markers and the presence of pro-inflammatory T cells and pro-fibrotic macrophages in the kidneys of mice. MCC950 also limited renal fibrosis and microalbuminuria, and reduced systolic and diastolic blood pressure by ~10 mmHg. Collectively, these findings confirm the key role of the NLRP3 inflammasome in the development of hypertension and renal inflammation and highlight it as a promising target for small-molecule inhibitors.

In conclusion, this thesis has provided further evidence that the NLRP3 inflammasome and IL-18 are key mediators of the renal inflammation and dysfunction that accompanies and ultimately exacerbates hypertension. Moreover, it has provided insights into new approaches to inhibit this system – including direct targeting of the NLRP3 inflammasome with a small molecule inhibitor and inhibition of inflammasome expression with NO donor drugs – which might be exploited as future therapies for hypertension.

Lay Description

High blood pressure affects approximately one third of the world's adult population and is a major cause of heart attacks, strokes and kidney disease. Alarmingly, many patients with hypertension are not treated effectively by currently-available blood pressure lowering drugs, highlighting the need for more research to identify new ways of treating the condition. Here, we have shown that a key component of the immune system known as the 'inflammasome' is switched on in the kidneys during hypertension and contributes to high blood pressure. By blocking the actions of the inflammasome with newly identified drugs, we reduced blood pressure and prevented renal damage in hypertensive mice. Hence, this project offers new hope to patients struggling to manage their blood pressure.

Publications during enrolment

Manuscripts as primary author

1. **Y.H. Ling**, S.M. Krishnan*, C.T. Chan, H. Diep, D. Ferens, J. Chin-Dusting, B.K. Kemp-Harper, C.S. Samuel, T.D. Hewitson, E. Latz, A. Mansell, C.G. Sobey, G.R. Drummond, Anakinra reduces blood pressure and renal fibrosis in one kidney/DOCA/salt-induced hypertension, Pharmacol. Res. 116 (2017) 77–86. doi:10.1016/j.phrs.2016.12.015.

2. S.M. Krishnan, **Y.H. Ling***, D. Ferens, C.T. Chan, H. Diep, M.M. Kett, C.S. Samuel, A.A.B. Robertson, M.A. Cooper, K. Peter, E. Latz, A. Mansell, C.G. Sobey, G.R. Drummond, A. Vinh, Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage and dysfunction in salt-sensitive hypertension [Unpublished], Cardiovasc. Res. (n.d.).

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2. M.A. Evans, H.A. Kim, **Y.H. Ling**, S. Uong, A. Vinh, T.M. De Silva, T. V. Arumugam, A.N. Clarkson, G.R. Zosky, G.R. Drummond, B.R.S. Broughton, C.G. Sobey, Vitamin D3 Supplementation Reduces Subsequent Brain Injury and Inflammation Associated with Ischemic Stroke, NeuroMolecular Med. 20 (2018) 147–159. doi:10.1007/s12017-018-8484-z.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes (1*)* original papers published in peer reviewed journals and (1*)* submitted publications. The core theme of the thesis is the role of inflammasomes, IL-1β, IL-18, and nitric oxide in inflammation and hypertension. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Pharmacology at Monash University under the supervision of Professor Grant Drummond.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 3 and Chapter 6, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) and % of contribution	Nature of Co-author's contribution & Monash student status	
3	Anakinra reduces blood pressure and renal fibrosis in one kidney/DOCA/salt-induced hypertension despite having minimal effect on renal inflammation	Published	55%. Performed DOCA/salt surgeries, BP measurements, real-time-PCR, Flow cytometry& immunohistochemistry	40%. Dr Shalini Murali Krishnan		Y
				<1%. Dr Christopher Te-Lee Chan	Assisted with flow cytometry experiments	Y
				<1% Mr Henry Diep	Assisted with immunohisto chemistry	N
				<1% A/Prof Chrishan S Samuel		N
				<1% Prof Timothy D Hewitson		N
				<1% Dr Ashley Mansell	Provided intellectual advice for experimental design and assistance with editing of manuscript	N
				<1% Prof Christopher G Sobey		N
				5% Prof Grant R Drummond	Conception and design of experiments, and assistance with drafting and editing manuscript	N

6	Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage and dysfunction in salt-sensitive hypertension	Submitted	45%. Assisted with Flow Cytometry experiments, Conducted immunohistochemistry experiments and real-time PCR experiments. Provided intellectual advice for design and assistance with editing of manuscript	45% Dr Shalini Murali Krishnan	Performed 1K/DOCA/salt surgeries, tail cuff measurements, conducted flow cytometry experiments	Y
				<1% Ms Dorota Ferens	Conducted radiotelemetry experiments	N
				<1% Dr Christopher Te-Lee Chan	Assisted with flow cytometry experiments	Y
				<1% Mr Henry Diep	Assisted with immunohistochemistry experiments	N
				<1% Dr Michelle M Kett	Provided intellectual advice and assisted with radiotelemetry experiments	N
				<1% A/Prof Chrishan S Samuel	Provided intellectual advice and assisted with immunohistochemistry experiments	N
				<1% Dr Avril AB Robertson	Provided intellectual advice and provided research tools (MCC950)	N
				<1% Prof Matthew A Cooper		N
				<1% Prof Karlheinz Peter	Provided intellectual advice	N
				<1% Prof Eicke Latz	Provided intellectual advice	N
				<1% Dr Ashley Mansell	Provided intellectual advice	N
				<1% Prof Christopher G Sobey	Provided intellectual advice	N

				5% Prof Grant R Drummond	Conception and design of experiments and assistance with drafting and editing of manuscript	N
				5% Dr Antony Vinh		N

*If no co-authors, leave fields blank

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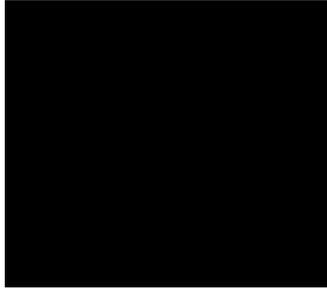
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

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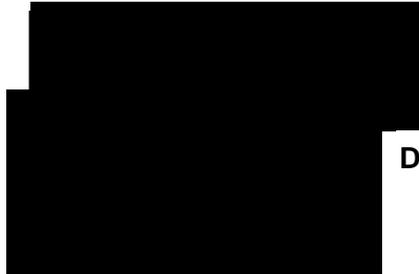
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At the end of a four year PhD program, I find myself with an incredibly extensive list of people in which to thank with not enough words to thank them with. That being said, I will attempt to impart on the reader of this acknowledgements the level of gratefulness I have for these people.

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P.S. Taking from another person, I just realised that a person who never usually gets thanked in these acknowledgements is the writer of the acknowledgements. I should say I am grateful to my past self for persevering long enough to complete a PhD candidature.

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List of Abbreviations

1K/DOCA/salt	One kidney/deoxycorticosterone acetate/salt
1K/placebo	One kidney/placebo
AIM2	Absent in melanoma 2
AP-1	Activator protein-1
ARC	Animal Research Centre
ARL	Animal Research Facility
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BMDMs	Bone marrow-derived macrophages
BP	Blood pressure
CARD	Caspase activation and recruitment domain
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
COL1A1	Collagen type 1 alpha 1
COL3A1	Collagen type 3 alpha 1
COL5A1	Collagen type 5 alpha 1
DAMPS	Danger-associated molecular patterns
eNOS	Endothelial nitric oxide synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-18	Interleuekin-18

IL-18BP	Interleukin-18 binding protein
IL-18R1	Interleukin-18 receptor Type 1
IL-18RAP	Interleukin-18 receptor accessory protein
IL-1R1	Interleukin-1 receptor Type 1
IL-1RA	Interleukin-1 receptor antagonist
IL-1β	Interleuekin-1β
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor associated kinase
JNK	Jun amino-terminal kinase
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MARP	Monash Animal Research Facility
mmHg	Millimetres of mercury
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NLRP3	NOD-like receptor family pyrin domain-containing protein 3
nNOS	Neuronal nitric oxide synthase
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RAG1	Recombination activating gene 1
ROS	Reactive oxygen species
SPF	Specific pathogen free

TEC	Tubular epithelial cells
Th	T helper cell
T_{regs}	T regulatory cell
VCAM	Vascular cell adhesion molecule 1

Chapter 1:

General Introduction

Introduction

Hypertension is a chronic medical condition classified by the American Heart Association (AHA) as systolic and diastolic arterial blood pressures being elevated above 130 on 80 mmHg, respectively [1]. It is a major risk factor for debilitating diseases such as myocardial infarctions and strokes which are leading causes of death and morbidity worldwide [2,3].

The incidence of hypertension in the population increases with age in both males and females, with around 50% of the population having hypertension by the age of 55 [4]. The life time risk of developing hypertension is around 90% in both males and females, while the lifetime probability of being put on an antihypertensive therapy is around 60% [5]. Current antihypertensive therapies such as angiotensin-converting enzyme (ACE) inhibitors, β-adrenergic receptor antagonists, Angiotensin (Ang) II Type 1 receptor antagonists, and diuretics (thiazides) tend to only treat the primary symptom of hypertension – elevated blood pressure. Hence, even with the widespread use of antihypertensive medications, in many patients, the disease is still poorly managed, and it is estimated that up to 20% of patients display "resistant hypertension" [6]. This is defined as being unable to achieve systolic and diastolic blood pressures under 140 and 90 mmHg, respectively, despite the use of optimum doses of three antihypertensives including a diuretic [7,8]. Clearly, current therapeutic approaches do not adequately treat the disease in all patients, highlighting the urgent need for a more comprehensive understanding of the disease mechanisms of hypertension in the hopes of finding new targets for more effective therapies.

1.1 Auto-immunity and inflammation

Autoimmune diseases are a group of diseases associated with dysregulation of the immune system resulting in the host's own immune system mounting an attack against what is generally regarded as 'self' [9]. This can result in a number of diseases, with prominent ones including rheumatoid arthritis [10], gout [11] and systemic lupus erythematosus [12].

Recent evidence suggest that, in many respects, hypertension might also be recognised as a chronic low-grade autoimmune disease [13–17].

The mammalian immune system is comprised of two major arms, the innate immune system and the adaptive immune system. The innate immune system is responsible for first line defence against pathogens or host-derived danger signals that may arise following tissue and cellular injury. In addition, the innate immune system is responsible for activating the adaptive immune system, which is responsible for long-term protection against known pathogen and danger signals [18,19]. The innate immune system is comprised of immune cells (also known as leukocytes) such as mast cells, macrophages, dendritic cells, neutrophils, basophils, and eosinophils. Their role in the initiation of the immune response can be considered quite generic, in that they respond to a wide range of pathogenic or viral organisms by releasing molecular signals to initiate inflammation [18,19].

Inflammation involves vasodilatation and an increase in blood flow to the area of infection or injury, increased metabolic activity, and the release of mediators such as cytokines, adhesion molecules, and chemokines that facilitate the influx and activation of immune cells to the local area [20]. Recruited cells usually include cells of the adaptive immune system, also known as lymphocytes, which include T cells and B cells. T cells can be further subdivided into T helper cells (T_h), Cytotoxic T (T_c) cells, regulatory T cells (Tregs) and memory T cells. Depending on their effector function and the suite of cytokines they release, T_h cells can be further subdivided into either T_h1 or T_h2 subsets. T_h1 responses are generally regarded as "pro-inflammatory" due to being associated with amplification of the initial inflammatory response established by the innate immune system in order to rapidly remove infiltrating pathogens. By contrast, T_h2 responses are often regarded as "anti-inflammatory" as they are associated with suppression of T_h1 responses and a shift towards tissue repair. Another subset of T_h cells known as T_h17, which are defined by their ability to produce interleukin-17 (IL-17), have also been shown to play a pro-inflammatory role in several autoimmune diseases including rheumatoid arthritis and psoriasis [21–24].

Although acute inflammatory responses are important for the removal of pathogens and damaged cells to facilitate the healing process after an infection or injury, chronic inflammation can lead to progressive tissue damage. This can lead to organ dysfunction and subsequently contribute to the pathophysiology of diseases [12,18,25–28]

1.2 Evidence for inflammation in clinical hypertension

There is a growing body of evidence to suggest that hypertension is a chronic, low-grade inflammatory condition. Clinical studies have shown that there is a positive association between systolic blood pressure and circulating levels of C-reactive protein, a protein that is produced in the liver in response to pro-inflammatory stimuli. Such studies have also shown that patients who are pre-hypertensive (120-129 mmHg systolic BP, 80-89 mmHg diastolic BP) have significantly elevated C-reactive protein compared to people with normal blood pressure [29,30], indicating that inflammation may be present from early on in the disease process. Clinical studies have also shown that in patients with auto-immune diseases such as rheumatoid arthritis, gout, and lupus, hypertension is frequently observed as a comorbidity [31–34]. Clinical and pre-clinical studies show that hypertensive patients and animal models display increased activity of the 'classical' pro-inflammatory transcription factor NF-κB [35,36]. NF-κB is known to be a key factor in the transactivation of numerous pro-inflammatory molecules including the adhesion molecules; intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [37], chemokines such as chemokine (C-C motif) ligand 5 (CCL5) and chemokine (C-C motif) ligand 2 (CCL2) [38] and pro-inflammatory cytokines such as interleukin 6 (IL-6) [39], IL-17 [25], IL-1β and IL-18 [40]. And indeed, all of these inflammatory mediators have been shown to be elevated in the blood and various tissues in both clinical and preclinical models of hypertension [41–45]. As direct evidence of a cause-effect relationship between inflammation and hypertension, several pre-clinical studies have shown that blocking the actions of these pro-inflammatory molecules can reduce many of the pathophysiological processes that accompany hypertension. For example, compared to wild-type mice, mice lacking IL-6

displayed a reduced pressor response to angiotensin II, as well as less renal damage and fibrosis [46,47]. Results were similar for mice lacking IL-17, which again were found to be protected against the pressor effects of angiotensin II, as well as the vascular function and T cell infiltration of the aorta [48]. Finally, mice administered with an antagonist that blocks the action of CCL2 and its chemokine receptor, CCR2, had reduced systolic blood pressure and macrophage infiltration in the aorta after deoxycorticosterone acetate/salt treatment [49].

1.3 Inflammasomes

Inflammasomes are multimeric enzyme complexes that regulate the activity of the cytokines IL-1β, IL-18 [50–53], IL-33 and IL-37 [54–56], by catalysing their processing from 'pro' uncleaved forms to 'mature' cleaved forms. In the case of IL-1β, IL-18 and IL-37, the cleaved form is the biologically active form. The opposite is true for IL-33, where processing by caspase-1 leads to a reduction in the cytokine's activity [50–56]. While it was first thought that inflammasomes were exclusively expressed in myeloid cells, such as macrophages and neutrophils, it is now known that they are present in multiple cell types [57]. For example, vascular endothelial cells [58], neuronal cells [59], dermal cells [60] and, of likely relevance to this project, renal tubular epithelial cells [61], are now known to express inflammasome components suggesting that these cells may play an important role in the initiation of the inflammatory response as part of the innate immune system.

Several isoforms of inflammasomes have been described each differing from one another mainly by the type of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) they contain [62]. Based on their N-terminus domain, NLRs can be classified into four subtypes including those that contain (1) an acidic transactivation domain (NLRA); (2) a pyrin domain (NLRP); (3) a caspase-recruitment domain (NLRC) and (4) a baculovirus inhibitor repeat domain [62]. Other inflammasomes described include the pyrin and HIN domain-containing protein (PYHIN) family members, absent in melanoma 2 (AIM2) and

interferon-γ-inducible protein 16 (IFI16). Both AIM2 and IFI16 lack an NLR to bind DNA, instead containing a HIN domain for binding DNA and a pyrin domain for the recruitment of the adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) [63].

The NLRP3 inflammasome complex contains the NLRP3 protein, an adaptor ASC protein and pro-caspase-1. The NLRP3 protein consists of an effector N-terminus pyrin domain, which is responsible for recruiting the adaptor protein ASC. It also contains a central NACHT or nucleotide binding domain (NBD), which is responsible for oligomerization of the complex, as well as a leucine-repeat rich (LRR) domain, which is responsible for ligand detection [62,64,65]. The ASC protein consists of a pyrin domain at its N-terminus and a caspase-recruitment domain (CARD) at its C-terminus. When activated, the pyrin domain on the NLRP3 protein associates with ASC. The CARD domain on ASC also associates with the CARD domain on pro-caspase-1 [51,52]

Activation of the NLRP3 inflammasome is a two-step process involving: (1) upregulation of transcription of the NLRP3 inflammasome components, also known as "priming" of the inflammasome; and (2) NOD-like receptor (NLR) dependent activation of inflammasome assembly [50,51]. Upon receiving these two signals, NLRs recruit the adaptor protein, ASC, and pro-caspase-1, and this clustering of the inflammasome complex leads to the auto-cleavage of pro-caspase-1 into active caspase-1 (Figure 1).
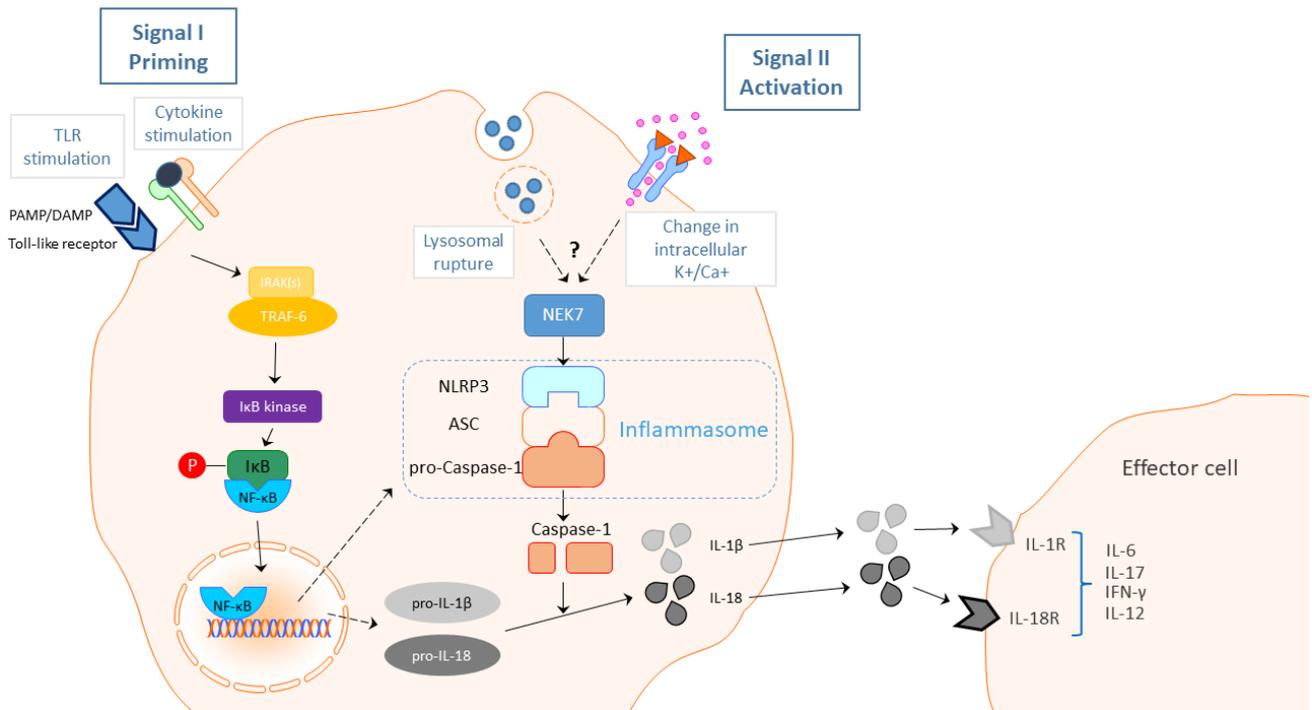
Figure 1: Pathway of the transcriptional priming and activation of the NLRP3 inflammasome. The NLRP3 inflammasome consists of the NLRP3 protein, an adaptor ASC protein and pro-caspase-1. Activation of the NLRP3 inflammasome is a two-step process involving: (1) transcriptional upregulation of NLRP3 inflammasome components in response to Signal I; and (2) clustering of the inflammasome complex in response to Signal II. This leads to auto-cleavage of pro-caspase-1 into active caspase-1 which subsequently cleaves the inactive pro-forms of IL-1β and IL-18 into their active forms.

1.4 Priming and activation mechanisms of the inflammasome

It is now known that many signals, both intrinsic and extrinsic, are able to prime and activate the inflammasome. These signals can be broadly classified under the terms pathogen-associated molecular patterns (PAMPS) and host-derived danger-associated molecular patterns (DAMPS). PAMPS are small distinguishing molecular patterns expressed by a wide variety of pathogens, while DAMPS are signals that indicate disruption to normal cell or host function [62]. Detection of these signals by the host occurs via pattern recognition receptors (PRR) [66–69]. Like the signals they detect, there exists a wide range of PRRs that are able to detect the multitude of signals that exist, with certain PRRs being more specific for certain signals than others. PRRs may be expressed on the plasma membrane to detect extracellular signals, or in the cytosol to detect intracellular signals. One example of a family of membrane-bound PRRs is the Toll-like Receptor (TLR) family [19]. Initially discovered in *Drosophila,* TLRs were first characterised to be important in the developmental biology of the dorso-ventral axis of the embryo [70]. A loss-of-function allele in the Toll gene resulted in dorsalisation of the embryo whereas a gain-in-function version of the allele resulted in ventralisation [70]. It was later found that these same receptors were important in the activation of the innate immune system in *Drosophila* by recognising PAMPS that were expressed by infectious organisms such as viruses and bacteria [71]. Indeed, it was subsequently found that TLRs were expressed in non-mammalian and mammalian species each able to detect a different suite of PAMPS [19]. For example, TLR4, which is ubiquitously expressed in mammalian species was discovered to be important in recognising lipopolysaccharide (LPS) [72], a molecule expressed in the outer membrane of gram-negative bacteria. TLR4 is also able to detect certain DAMPs such as heparan sulphate [73] and hyaluronic acid fragments, molecules that are important in the formation of the extracellular matrix, which when damaged may be indicative of abnormal host function [74]. Other TLRs include TLR2, which detects lipoteichoic acid, expressed in the outer membrane of gram-positive bacteria [75]; TLR3, which recognises double-stranded

RNA such as polyinosinic:polycytidylic acid (Poly I:C) from viruses [76]; TLR5, which detects bacterial flagellin [77]; TLR7, which is activated upon detection of single-stranded RNA from viruses [78]; and TLR9, which recognises single stranded DNA such as CpG oligodeoxynucleotide from bacteria and DNA viruses [79]. Cytokines are also able to act as DAMPs and activation of their signalling pathways leads to transcriptional upregulation of the inflammasome. Cytokines that are able to do this include TNF-α and IL-1β. Indeed, the IL-1R1 shares a domain similar to those found in TLRs known as the Toll/IL-1 receptor domain (TIR) [18].

Like priming signals, the list of danger signals that are now known to cause oligomerisation and activation of the NLRP3 inflammasome complex is quite extensive. For example, such signals include (but are not limited to) PAMPs such as the bacterial pore-forming toxin, nigericin [50] and viral infections [40,80]; endogenous danger signals such as extracellular glucose [81], extracellular ATP [82], Amyloid β [83], and hyaluronan [74], which is released from the extracellular matrix after injury. Crystalline substances such as uric acid crystals [84], silica crystals [85] and cholesterol crystals have also been identified as powerful inducers of NLRP3 inflammasome activity [86]. However, despite the many signals that are known to activate the inflammasome, the precise molecular mechanism by which these signals activate the inflammasome remains elusive. Many mechanisms have been proposed including lysosomal destabilisation, changes in intracellular calcium levels, and the formation of large nonspecific membrane pores and subsequent potassium efflux [50,87]. Regarding the latter, potassium release seems to accompany NLRP3 inflammasome activation irrespective of the activating stimulus suggesting that it may be a common pathway for inflammasome activation [88]. However, there is no direct evidence that NLRP3 directly senses these ion changes, and thus, it is possible that potassium efflux may be associated with other cellular events that are actually responsible for activating the inflammasome.

Recently, a role of NEK7 in the recognition of danger signals by the NLRP3 inflammasome has been suggested [89–91]. NEK7 belongs to a family of serine/threonine-protein kinases known as 'Never In Mitosis A' (NIMA)-related kinases [91]. These kinases seem to be important in the regulation of the cell cycle process by controlling the timing of mitotic entry, spindle organisation, chromatin condensation and cytokinesis [91]. A recent study has shown that NEK7 interacts directly with the NLRP3 subunit, independent of ASC and pro-caspase-1 [90]. This interaction was shown to be essential for activation of the NLRP3 inflammasome such that deficiency of NEK7 resulted in the abolishment of inflammasome activation in response to several diverse activators [90]. Furthermore, macrophages that have the CAPS-associated NLRP3 mutant which do not require potassium efflux for activation still require NEK7 for caspase-1 activation. Collectively, these findings suggest that NEK7 operates downstream or independently of potassium efflux in the pathways leading to inflammasome activation [90,91].

1.5 Actions of the IL-1 cytokine superfamily

IL-1β and IL-18 are members of the pro-inflammatory IL-1 cytokine superfamily [92]. Considered to be early initiators of the immune response, activity of these cytokines leads to the upregulation and release of other pro-inflammatory cytokines such as IL-6 [39], IL-17 [93], IL-12 [94] and Interferon-γ (IFN-γ) [95]. As mentioned previously, circulating levels of these cytokines are known to be elevated in patients with hypertension. While IL-1β and IL-18 were initially thought to be mainly released from cells which play a role in the early initiation of the immune system, such as macrophages and monocytes, it is now known that these cytokines can be produced by non-immune cell types [96]. Cell types that are potentially relevant to the development of hypertension and which express these cytokines include vascular endothelial cells and renal tubular epithelial cells [97,98].

The pro-inflammatory actions of IL-1β and IL-18 are mediated by stimulation of their cognate cell surface receptors, namely, the IL-1 receptor Type 1 receptor (IL-1R1) and the IL-18

Type 1 receptor (IL-18R1) respectively [54,99]. The binding of IL-1β and IL-18 to their receptors leads to the recruitment of several distinct yet homologous accessory proteins which are required for downstream signalling. In the case of IL-1R1, stimulation leads to recruitment of the IL-1 receptor accessory protein (IL-1RAP) whereas IL-18R1 stimulation leads to recruitment of the IL-18 receptor accessory protein (IL-18RAP) [100]. In each case, association of the relevant accessory protein with the receptor complex leads to the recruitment of intracellular adaptor proteins including myeloid differentiation factor 88 (MyD88), IL-R-associated kinase (IRAK), and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6). These adaptor proteins then initiate pro-inflammatory signalling cascades which result in the activation of mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK) and p38 mitogen-associated protein kinase (MAPK), and in turn stimulation of transcription factors such as NF-κB and activator protein-1 (AP-1). These transcription factors ultimately bind to the promoter regions of genes encoding pro-inflammatory molecules such as IL-6 and IFN-γ [101].

The above paragraph presents a simplistic description of the actions of IL-1β and IL-18. Indeed, the actions of these cytokines are regulated not only by their concentration within tissues or the expression profile of their respective receptors, but also by the presence of several inhibitory molecules which exist to prevent the system from becoming excessively activated. In the case of IL-1β, there exist a decoy receptor known as the IL-1R Type II (IL-1RII) that is unable to stimulate the signalling pathway, hence reducing availability of IL-1β to bind to IL-1R1 [51,102]. There also exists an endogenous IL-1R1 antagonist known as interleukin-1 receptor antagonist (IL-1Ra), which competes for binding at IL-1R1 to reduce IL-1β activity [103]. IL-18 is similarly counter-regulated by the activities of a molecule known as the IL-18 binding protein (IL-18BP). IL-18BP is constitutively secreted from mononuclear leukocytes and is able to bind to circulating IL-18 to prevent it from interacting with its receptor, thereby neutralising its effects [104].

Other cytokines are also able to either directly or indirectly manipulate the IL-1β and/or IL-18 signalling system. IL-33 and IL-37 are more recently identified members of the IL-1 family and have been shown to modulate the activity of other members of the IL-1 family [54,56]. In contrast to IL-1β and IL-18, the actions of IL-33 and IL-37 are anti-inflammatory [56,105,106]. For example, IL-33 triggers an anti-inflammatory response when bound to its cognate receptor, the interleukin 1 receptor-like 1 (IL-1RL1) receptor. This leads to recruitment of IL-1RAP and the modulation of NF-κB and MAP kinase pathways which stimulate the release of anti-inflammatory cytokines such as IL-5 and IL-13, which skew the immune system towards a Th2 response [105]. IL-37, like IL-33 has also been shown to primarily exert an anti-inflammatory effect by downregulating the expression of pro-inflammatory cytokines such as IL-1α, IL-6 and TNF-α and upregulating the expression of the anti-inflammatory cytokine, IL-10 [107]. Currently, there is relatively little information on how IL-37 mediates its anti-inflammatory effects. However, there is some evidence to suggest that IL-37 may not have its own cognate receptor, but rather binds to the IL-18R1 to promote the recruitment of a different set of accessory proteins such as IL-1R8 and IL-1R9, which direct IL-18R1 signalling along an anti-inflammatory pathway [56]. IL-37 has also been shown to have direct intracellular effects by acting as a transcription factor to directly affect gene expression of downstream cytokines [56,107]. Recent studies have also shown that IL-37 could possibly be mediating these effects by modulation of the Smad3 pathway [107,108]. The Smad3 pathway is known to regulate the expression of the cytokine, transforming growth factor beta (TGF-β) [109]. TGF-β has numerous effects on various immune cells including monocytes, macrophages, dendritic cells, T cells and B cells, but overall, its action results in suppression of local inflammation [110]. Studies have shown that Smad3 and IL-37 form a functional complex and by silencing the Smad3 pathway, the anti-inflammatory effects of IL-37 are reduced [107].
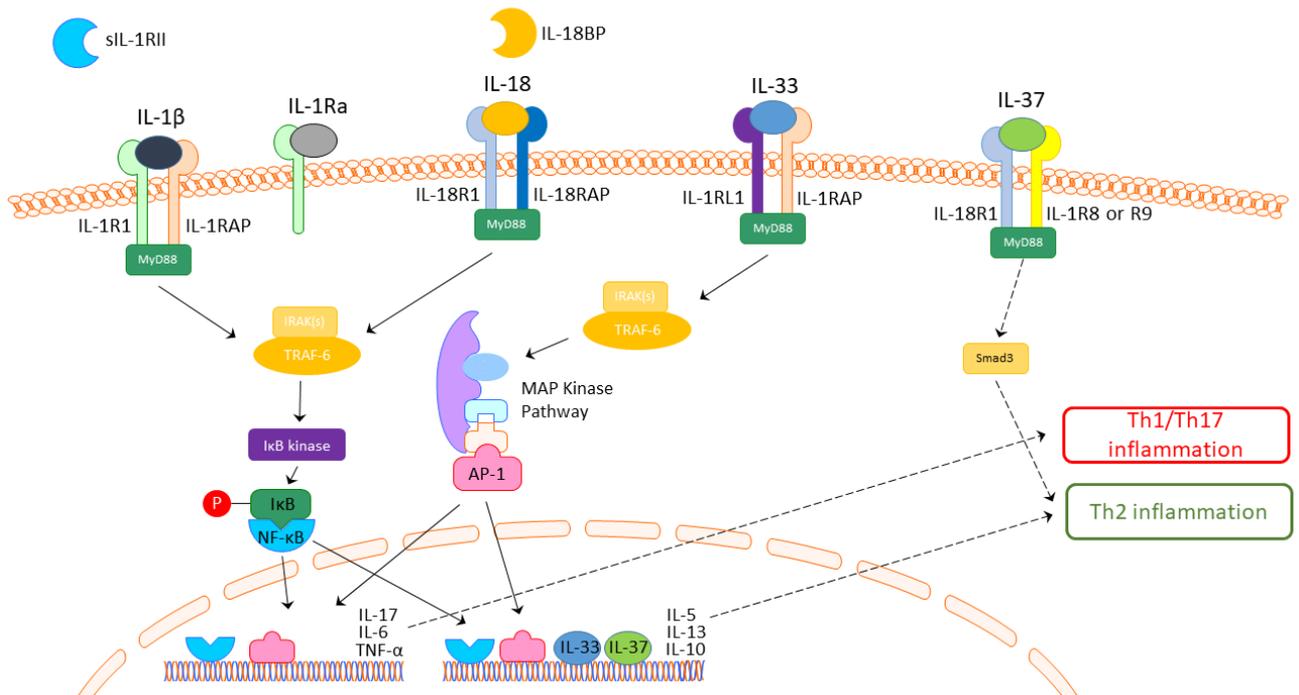
Figure 2: Signalling pathways and endogenous antagonists of the IL-1 cytokine family. Binding of IL-1β to IL-1R1 and IL-18 to IL-18R1 is facilitated by unique yet homologous accessory proteins, IL-1RAP, and IL-18RAP. Endogenous inhibitors include IL-1Ra, a competitive antagonist against IL-1β at the IL-1R1; IL-1RII, a decoy receptor for IL-1β, and IL-18BP, a binding for IL-18. Intracellular signalling pathways involving MyD88, MAP kinase pathways and phosphorylation of IκB leads to cellular relocalisation of transcription factors such as NF-κB and AP-1, which upregulate production of various cytokines. IL-33 binds to a unique receptor facilitated by the IL-1RAP which leads to the upregulation of a Th2 response. IL-37 seems to bind to the IL-18R1 in association with IL-1R8 or R9 which upregulates the Smad3 pathway leading to a Th2 inflammatory response.

1.6 IL-1β and IL-18 in hypertension

As previously mentioned, hypertension is now generally regarded as a chronic low grade inflammatory disease. Consistent with this, meta-analyses of clinical studies have shown that hypertensive patients display elevated circulating levels of the inflammasome-derived cytokines, IL-1β and IL-18 [44,45]. Pre-clinical studies have also shown that IL-1β and IL-18 have effects on the cardiovascular and renal system that may be consistent with a pro-hypertensive role. For example, *ex vivo* IL-1β treatment of resistance arteries from rats caused impairment of endothelium-dependent relaxation responses to acetylcholine [111,112] while potentiating vasoconstrictor response to phenylephrine [113]. For this latter effect, IL-1β had a greater effect in vessels from hypertensive mice suggesting that the condition may be associated with sensitization of the vasculature to IL-1β. It remains to be determined whether this is the result of increased expression of IL-1 receptors, or if other mechanisms are involved. Immune cells from hypertensive patients also seem to be primed to produce more IL-1β in response to angiotensin II, compared to those from normotensive patients [114,115]. This effect was reversible with either *ex vivo* or *in vivo* administration of an angiotensin AT1 receptor antagonist, highlighting the fact that T cell activity can be directly modulated by the renin angiotensin system (RAS) and implying that IL-1β may be a mediator of the end-organ inflammation seen in hypertensive patients in response to circulating angiotensin II. Furthermore, levels of the endogenous IL-1β inhibitor, IL-1Ra, have been shown to be increased in patients with essential hypertension compared to normotensive controls indicative of a compensatory response to offset elevated activity of the IL-1β signalling system in hypertensive patients [116].

Similar to IL-1β, IL-18 has also been shown to have direct effects on the vascular system that would be consistent with a pro-hypertensive role of this cytokine. For example, IL-18 was shown to promote the proliferation and migration of vascular smooth muscle cells in culture, both of which are key cellular processes in the vascular remodelling that occurs during hypertension [117,118]. These effects were mediated by activation of NADPH

oxidase (NOX)-derived reactive oxidative species (ROS) which in turn led to the activation of transcription factors including NF-κB and AP-1 [117]. Furthermore, siRNA-mediated knockdown of IL-18 reduced the proliferation of cultured vascular smooth muscle cells in response to angiotensin II. This finding is particularly interesting because it suggests that vascular smooth muscle cells themselves are capable of producing IL-18 and that it acts as a mediator of the vascular remodelling induced by angiotensin II [117]. Furthermore, IL-18 levels were shown to be directly correlated with intima-media thickness (a marker of future cardiovascular risk) of the carotid artery in patients with hypertension [119,120]. Finally, mice that overexpress IFN-γ, a cytokine that is produced downstream of IL-18 (indeed, IL-18 was originally known as IFN-γ inducing factor), displayed increased Ang II induced vascular endothelial dysfunction compared to wild-type mice. This was reversed in IFN-γ$^{-/-}$ mice which were partially protected against the same treatment [121].

More recently, our laboratory and others have provided direct evidence of a causative role for the inflammasome and its cytokine products, IL-1β and IL-18, in the development of hypertension [122,123]. Mice lacking the inflammasome adaptor protein, ASC, displayed a blunted hypertensive response to 1K/DOCA/salt treatment. This reduced pressor response was paralleled by reductions in the expression of several pro-inflammatory cytokines in the kidneys, including IL-6 and IL-17 as well as less infiltration of the kidneys by inflammatory immune cells [122]. Similar findings by our lab and others were obtained in a renin-dependent model of hypertension, where both ASC and NLRP3 knockout mice were shown to be protected against increases in BP and renal inflammation, suggesting that the inflammasome appears to play a role in the pathogenesis of hypertension irrespective of the stimulus [122,123].

The above findings highlight a causative role of the inflammasome in the pathogenesis and development of hypertension. However, it still remains to be determined whether intervention treatment with a pharmacological inhibitor of the NLRP3 inflammasome (similar to how the disease would be treated in the clinic) shows similar protective effects compared

to lifelong genetic deficiency. Furthermore, the relative contribution of the two main inflammasome-derived cytokines, IL-1β and IL-18, to the development of the disease remains to be determined. There exists a number of research tools which could allow for the addressing of these questions. Firstly, a recently developed small molecule inhibitor of the NLRP3 inflammasome, MCC950, has been shown to be highly selective and effective at treating other renal and inflammatory diseases [124,125]. Secondly, knockout mice that are selectively deficient in either IL-1β or IL-18 exist [126,127] and could be readily used to examine the role of these systems in the context of hypertension. Indeed, such methods will be employed in the current study. In addition, specific and selective pharmacological inhibitors of each of these cytokines have been identified and have the potential to be viable candidates for anti-hypertensive treatments.

1.7 Inhibitors of NLRP3 inflammasome activation

MCC950 is a recently identified small molecule inhibitor of the NLRP3 inflammasome. While the mechanism is currently unknown, it has been shown to specifically inhibit the NLRP3 inflammasome without affecting other inflammasome isoforms such as AIM2, IFI16, or NLRC4. It has been shown to potently inhibit IL-1β production by NLRP3 in both mouse and human macrophages with an IC50 of < 10 nM [124]. Furthermore, treatment with MCC950 in an experimental model of autoimmune encephalitis in mice resulted in reduced IL-1β, IL-6, and IL-17 production resulting in improved clinical score [124]. Treatment with MCC950 was also shown to reduce levels of IL-18 and improve the survival rate of mice with cryopyrin-associated periodic syndromes (CAPS) [124].

β-hydroxybutyrate is an endogenous ketone metabolite that was recently shown to prevent oligomerization of the NLRP3 inflammasome [128]. However, unlike MCC950, this mechanism is a result of actions that are upstream of inflammasome oligomerization and seems to inhibit the process of K^+ efflux, previously mentioned to be important in the activation of the NLRP3 inflammasome. Nevertheless β-hydroxybutyrate has been shown

to prevent IL-1β production in several mouse models of NLRP3-associated diseases including Muckle-Wells syndrome, familial cold auto-inflammatory syndrome and gout, resulting in reduced disease severity. [129].

Another potential inhibitor that could be investigated is a recently developed small molecule inhibitor of NLRP3 inflammasome activation, AC-201. While a lot is unknown about the structure of this inhibitor, its use has been approved for Phase II clinical trials for the treatment of gout [130,131].

1.8 Inhibitors of IL-1β signalling

As previously mentioned, there exists an endogenous inhibitor of IL-1β signalling known as the IL-1 receptor antagonist (IL-1Ra) [103]. IL-1Ra inhibits the actions of IL-1β, but not IL-18, by competing for binding at the native receptor for IL-1β, IL-1R1. Anakinra is a recombinant human IL-1R1 antagonist that has similar inhibitory properties to endogenous IL-1Ra [52]. Indeed, the synthetic antagonist shares almost 100% similarity in terms of both its 2-dimensional and 3-dimensional structures to the endogenous human protein except for an additional methionine residue at the N-terminus to increase its stability [132]. It is currently indicated for the treatment of the auto-inflammatory disease, rheumatoid arthritis [133], but has also been shown to be beneficial in animal models of other inflammatory diseases such as gout [134] and systemic lupus [135]. Due to its short half-life (4-6 hours) and poor oral bioavailability (i.e. must be administered subcutaneously via injection), patient compliance has been identified as an issue [133]. Furthermore, anakinra is primarily metabolised by the kidneys. Therefore, anakinra is contraindicated in patients with impaired kidney function, which is a potential issue in the context of hypertension [133].

Canakinumab is a high affinity human monoclonal neutralising antibody against IL-1β [136]. Due to its longer plasma half-life and better pharmacokinetic profile compared to earlier drugs such as anakinra, it has been approved by the U.S. Food and Drug Administration (FDA) for the use in several auto-inflammatory syndromes including Muckle-Wells

syndrome, familial cold auto-inflammatory syndrome and neonatal-onset multisystem inflammatory disease [136]. Interestingly, all these diseases are known as CAPS which are associated with a mutation in the NLRP3 gene resulting in it being more highly expressed in these patients [136]. Canakinumab was also taken into Phase III clinical trials to investigate its efficacy in chronic obstructive pulmonary disease, gout, and coronary artery disease (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study [CANTOS]) [137]. The findings from these studies were highly promising, in that treatment with 150 mg of canakinumab every three months resulted in a 31% reduction in deaths associated with heart attacks, strokes, and cardiovascular diseases combined. Canakinumab treatment was also associated with reduced serum levels of C-reactive protein, a marker of general inflammation. Yet, while this study highlights the potential of blocking IL-1β signalling in the treatment of cardiovascular diseases per se, no information was provided about the effect canakinumab had on hypertension status [137].

Although the above discussion highlights some of the clinical advantages of canakinumab over anakinra, there is currently no data on whether this mAb is effective at neutralising the mouse IL-1β homologue, either *in vitro* or *in vivo*. Hence for this reason, the current study utilised anakinra to investigate the role of IL-1β in hypertension.

1.9 Inhibitors of IL-18 signalling

In contrast to IL-1β, there are currently no IL-18 inhibitors that are approved for the treatment of inflammatory diseases in the clinic. However, inhibitors of IL-18 signalling do exist and include a human monoclonal IL-18 neutralising antibody and a human recombinant IL-18BP; both of which are currently being tested in clinical trials for Adult-onset Still's disease, a rare auto-inflammatory disease; rheumatoid arthritis; psoriasis; and Type II diabetes [104,138–140]. While there is still no data on the efficacy of these treatments, preliminary findings suggest that these inhibitors have excellent safety and tolerability profiles in healthy and diseased patients. More recently, Deng and colleagues

[141] described several candidates for small molecule inhibitors of IL-18. These inhibitors seem to directly interact with three distinct hydrophobic interfaces on the IL-18 protein that are responsible for protein-protein interactions with the IL-18R1 and IL-18BP. The authors also speculate that inhibition of these protein-protein interactions could result in disruption of the recruitment of the adaptor protein IL-18RAP into the tertiary complex, which inhibits downstream signalling. One of these molecules, NSC80734 was especially promising, displaying inhibition of IL-18-induced IFN-γ production in KG-1 human bone marrow-derived macrophages with a half maximal effective concentration (EC50) of ~250 nM [141]. Indeed, there is an urgent need for small molecule cytokine inhibitors given that peptide-based therapies still have major limitations including poor pharmacokinetic profiles and the propensity to induce immune responses. Therefore, it will be interesting to see the impact that these inhibitors may have on cardiovascular outcomes and hypertension.

1.10 Nitric oxide donors

Nitric oxide (NO) is a gaseous lipophilic free radical and an important signalling intermediate in many physiological and pathophysiological processes. It was initially discovered as the endothelium derived relaxation factor (EDRF) as a result of researchers trying to determine the mechanism by which an unknown agent released by the endothelium could regulate vascular tone in blood vessels [142]. Owing the powerful vasodilator properties of NO, NO donor compounds, which release NO upon bioconversion (in the case of glyceryl trinitrate) or solubilisation in aqueous solutions (in the case of sodium nitroprusside and spermine nonoate) are currently indicated for several cardiovascular conditions such as angina, acute myocardial ischemia and severe perioperative hypertension [143–145].

In addition to its powerful vasodilator effects, NO is also known to play a key role in the regulation of inflammation. Under normal physiological conditions, NO exerts an anti-inflammatory influence on the vascular wall by suppressing the expression of adhesion molecules on the endothelium and inhibiting several leukocyte functions including

neutrophil and monocyte adhesion, immune cell chemotaxis and superoxide generation [146,147]. However, in response to injury or inflammatory assault, pro-inflammatory cytokines can increased the expression of inducible nitric oxide synthase (iNOS), an enzyme that catalyses the formation of NO by converting the amino acid, L-arginine to L-citrulline [148]. This increase in expression results in NO production from inflammatory cells such as neutrophils and macrophages at much higher levels than what is observed during normal physiological conditions [149]. At higher concentrations NO paradoxically enhances the inflammatory response by promoting the formation of cytotoxic free radical molecules and by activating apoptotic pathways and tissue damage [150]. It is important to note that NO is also regulated by other nitric oxide synthases including endothelial NOS (eNOS) and neuronal NOS (nNOS) [148]. Unlike iNOS, eNOS and nNOS are constitutively expressed [151]. Furthermore, while all three isoforms of the enzyme require the binding of calmodulin, which is dependent on intracellular calcium levels, to function, iNOS is able to bind tightly to calmodulin even in the presence of low calcium concentrations. This results in higher and more sustained production of NO by iNOS compared to the other isoforms [151]. In summary, depending on the amount that is produced, NO can act either as an anti-inflammatory or a pro-inflammatory mediator.

NO mediates its (patho)physiological effects through two mechanisms. The best characterised of these involves activation of soluble guanylate cyclase (sGC), an enzyme that regulates the production of cyclic guanosine 3', 5'-monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) [152]. cGMP is an important second messenger in the cardiovascular system where it regulates vascular tone and platelet function [153,154]. It can also regulate other processes such as cell division and apoptosis [150]; nucleic acid synthesis [155]; ion channel conductance [156]; and glycogenolysis [157]. NO is also able to signal via S-nitrosylation of regulatory proteins in various biological systems. S-nitrosylation is the process whereby an NO group is covalently attached to cysteine residues on proteins. Thus, it is a form of post-translational protein modification [158]. This chemical

modification induces conformational changes in proteins which can modulate their function or, in some cases, cause misfolding and render the protein dysfunctional. Indeed, S-nitrosylation is implicated in many neurodegenerative diseases such as Parkinson's disease [159], Alzheimer's disease [160] and Lewy body dementia [161,162] as a result of an accumulation of misfolded proteins in different areas of the brain leading to stress-induced neuronal death. Other diseases in which S-nitrosylation has been implicated include cancer, type II diabetes and asthma [163]. However, in contrast to the evidence for its contribution in certain diseases, S-nitrosylation has shown to be beneficial in some conditions such as ischaemic reperfusion injury after stroke, bronchopulmonary dysplasia, and acute lung injury by reducing the levels of inflammation seen in these conditions [163].

The above section highlights the diverse effects that nitric oxide has on many physiological and pathophysiological state. As such, nitric oxide may be viewed as a double-edged sword depending on not only its local concentration but also on the specific proteins it modifies or signal transduction pathways it activates. Of interest to the current study, little is known about the mechanism by which nitric oxide is able to exert an anti-inflammatory effect in certain disease settings. Hence, we will investigate whether inhibition of inflammasome activity may provide at least part of the explanation.

Conclusion

In summary, elevated levels of IL-1β and IL-18 are known to be associated with the development of hypertension. Furthermore, there is now direct evidence that genetic deficiency of the NLRP3 inflammasome blunts the development of hypertension and its associated end organ damage. However, it still remains to be determined whether pharmacological inhibition of the NLRP3 inflammasome has similar protective effects to genetic deficiency. Furthermore, the relative contribution of IL-1β vs IL-18 in the pathophysiology of hypertension remains unknown. Hence, this thesis will first determine whether pharmacological inhibition of the NLRP3 inflammasome has similar effects to

genetic deficiency in the treatment of hypertension. This thesis will also focus on determining directly the roles that IL-1β and IL-18 each play in the development of hypertension by utilising specific inhibitors or genetic knockout models. The thesis will also focus on determining the role that nitric oxide may play in the regulation of these IL-1 cytokines. Therefore, the aims of this thesis are as follows:

1. To investigate if pharmacological inhibition of IL-1β signalling with anakinra reduces renal inflammation and blood pressure in hypertension

2. To determine the contribution of IL-18 to renal inflammation and elevated blood pressure in hypertension and whether genetic deletion blunts the development of hypertension

3. To determine the mechanism of the anti-inflammatory effects of nitric oxide by investigating the role of NO donors in the regulation of the NLRP3 inflammasome

4. To determine if pharmacological inhibition of the NLRP3 inflammasome with MCC950 reduces renal inflammation and blood pressure in hypertension

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Chapter 2:

General Methods

Chapter 2: General Methods

2.1 Ethics Approval

The studies were approved by the Monash University Animal Research Platform Animal Ethics Committee (approval numbers MARP/2013/043 and MARP/2015/034) and were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition (2013).

2.2 Animals

Male C57BL/6J mice or IL-18$^{-/-}$ mice of a C57BL/6J background and age 10-12 weeks were obtained from Monash Animal Research Platform (Monash University, Clayton, Australia), or the Animal Resources Centre (Canning Vale, Western Australia). Mice were transported in high barrier SPF containers and housed in the Monash Animal Research Laboratories (ARL) in OptiMICE® cages (484 cm$_2$ x 13 cm) on a 12 h light-dark cycle. Mice were provided with ad libitum access to normal chow and drinking water (unless stated otherwise).

2.3 Blood pressure measurements

Systolic blood pressure (BP) was measured via tail cuff plethysmography or radiotelemetry. For tail cuff plethysmography, systolic BP was measured using an MC4000 multi-channel BP analysis system (Hatteras Instruments, USA). Mice were placed in a restraint on a heated platform (40°C) and an inflatable cuff was placed around the base of the tail. Light from an LED source is passed through the tail to a sensor allowing detection of the pulse. Systolic BP is defined as the pressure required to inflate the cuff to the point where blood flow to the tail is first terminated as detected by a change in the light signal. Mice were trained on the machine every day for three days prior to induction of hypertension. Systolic BP was then measured prior to surgery on day 0, afterwards on day 3, 7, 10, 14, 17 and 21 for Chapter 3 & 6 and weekly for Chapter 4.

For radiotelemetry, animals were surgically implanted with a radio telemetry probe (Model TA11PA-C10, Data Sciences International, USA). Briefly, this first involved placing mice

under general anaesthesia using an isoflurane machine (0.4 L/min, 2.5% maintenance). A midline incision is made which allows the observation of the left carotid artery. The radiotelemetry transmitter catheter is inserted into the left carotid artery with the probe body subcutaneously placed in the right flank. The wound site was then closed with surgical sutures. Mice were allowed to recover for 10 days prior to induction of hypertension. Systolic and diastolic BP, heart rate, and mean arterial BP were measured for 24 h in freely moving animals for at least 3 days prior to and throughout the treatment period. NB. The radiotelemeter implantation and data acquisition were performed by Ms Dorota Ferens

2.4 Model of hypertension

Mice were placed under general anaesthesia using an isoflurane machine (0.4 L/min, 2.5% maintenance). An incision was made through the skin on the left side of the abdomen to reveal the superficial muscle layer. After locating the spleen by observation of a dark band under the muscle layer, a small incision was made in this location to expose a small area of the abdominal cavity. The left kidney was then identified and its renal artery was tied twice with 4 mm surgical sutures. The left kidney was then excised from the animal. After removal of the kidney, the muscle incision was closed with surgical sutures. Before closure of the skin incision, blunt dissection was used to create a small subcutaneous pouch in the left scapular region into which a deoxycorticosterone (DOCA)-releasing pellet (2.4 mg/d; Innovative Research of America, USA) or a placebo pellet was inserted. Bupivicaine (0.5%, Pfizer, USA) was applied to the area to minimize post-operative pain. The skin layer was then closed with tissue clips and mice were allowed to recover for 1 h under close observation before being returned to their home cage. Mice treated with DOCA also had their drinking water replaced with 0.9% saline, while those treated with placebo pellets continued to receive normal drinking water. Mice were monitored daily for 3 days after surgery and twice-weekly thereafter for the remainder of the 21 day treatment protocol.

2.5 Chapter 3 Anakinra intervention protocol

Ten days after surgery to induce hypertension, some mice were further treated with anakinra (25-75 mg/kg/d; Amgen, USA) or vehicle (0.9% saline) for the remaining 14 days of the experiment via daily *i.p.* injections of 100 µl volume. A cohort of normotensive mice were also treated with anakinra or vehicle commencing 10 days after surgery.

2.6 Chapter 6 MCC950 intervention protocol

Similar to the previous intervention treatment, ten days after surgery to induce hypertension, some mice were further treated with MCC950 (10 mg/kg/d; obtained from Dr AAB Robertson, University of Queensland) or vehicle (0.9% saline) for the remaining 14 days of the experiment via implantation of an micro-osmotic minipump (Alzet, USA). Briefly, this involved placing mice under general anaesthesia using an isoflurane machine 0.4 L/min, 2.5% maintenance). A small incision was made at the nape of the neck and blunt dissection was used to make a subcutaneous pocket into which the minipump containing either MCC950 or saline was implanted. The incision was closed using a wound clip. A cohort of normotensive mice were also treated with MCC950 or vehicle commencing 10 days after surgery.

2.7 Assessment of kidney function using metabolic cages

Mice were housed individually in mouse-specific metabolic cages (Scientific Glassware, Faculty of Medicine, University of Melbourne) for 24 h intervals, allowing the volume of water/saline intake and urine production over that time to be accurately measured. Prior to taking any measurements, all mice were habituated by placing them in the metabolic cages for 8 h once and then for 24 h on two further occasions. Three separate measurements were then obtained across the 1K/DOCA/salt plus MCC950 or vehicle treatment period including: on day -1 to obtain baseline parameters; on day 9 to assess the impact of 1K/DOCA/salt treatment on kidney function, and finally on day 20 to assess the impact of MCC950 versus vehicle treatment. Once volume measurements were obtained, the collected urine was stored at -80°C for analysis of osmolality (Advanced Osmometer 2020;

Advanced Instruments, USA), Na$^+$ concentration (RAPIDChem744, Siemens, Germany) and albuminuria (Albuwell M, Exocell, USA).

2.8 Flow Cytometry

2.8.1 Isolation of leukocytes from kidneys and spleen

At the end of the treatment period, mice were killed by carbon dioxide asphyxiation or isoflurane overdose and perfused through the left ventricle with 0.2% clexane (400 IU, Sanofi Aventis, Australia) in RNAse-free phosphate-buffered saline (PBS). The spleen and right kidney were excised from the animal. Half of the kidney was set aside for flow cytometry or immunohistochemistry, while the other half was further divided into two equal portions, each of which were snap frozen in liquid nitrogen.

Half kidney samples were minced with fine scissors and enzymatically digested by incubation for 30-45 min 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). Spleen samples were minced with fine scissors without digestion. Digested kidney samples and spleen samples were passed through a 70 µm cell strainer filter. Kidney samples were subjected to a Percoll^{TM} gradient spin to further purify samples. This was achieved by resuspension of the strained cells in 30% Percoll (GE Healthcare, Sweden) and underlaying 70% Percoll in PBS. Kidney samples were then centrifuged at 2500 RPM for 25 min at room temperature in a Beckman Allegra 6R Centrifuge (Beckman Coulter, USA) with the brakes off. The cells at the interphase of the two density gradients were collected, washed in PBS and then resuspended in PBS containing 0.5% bovine serum albumin ("FACS" buffer). Spleen samples were subjected to osmotic lysis of red blood cells before being washed in PBS and resuspended in FACS buffer.

2.8.2 Antibody staining of extracellular markers

Cell suspensions were transferred onto 96 U or V-bottom plates and pelleted by centrifugation at 1500 RPM for 5 min at 4°C. Cells were then incubated with LIVE/DEAD®

Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific, USA; 1:1000 dilution in PBS) for 15 min after which time they were washed with FACS buffer and pelleted by centrifugation as previously described. All samples were ultimately stained for extracellular cell-specific markers by incubation in a cocktail of fluorophore-conjugated antibodies for 15 min at 4°C in the dark.

Along with samples, single-colour compensation were prepared from either spleen single cell suspensions or UltraComp eBeads® (eBioscience, USA) at a 1:2 dilution. Single-colour compensations prepared from spleen cells were incubated with the concentration of antibody used for samples whereas eBeads® were incubated with <0.125 µg (0.1-0.5 µl) of antibody in the conditions described above.

Following antibody staining, cell samples and single-colour compensations were washed and resuspended in 200 µl of FACs buffer. CountBright counting beads (Invitrogen, USA) were added to samples prior to flow cytometric analysis for normalisation and calculation of absolute numbers of cells in each samples. 7AAD was added to some samples 4-5 min prior to flow cytometric analysis for visualisation of live/dead cells. Flow cytometric analysis was performed on a BD LSR Fortessa™ (BD Bioscience, USA). Data were analysed with FlowJo Software (version 10.2, Tree Star Inc, USA). Cell numbers were normalised to CountBright counting beads and expressed as total cells per organ or as percentage of the parent cell population.

2.8.3 Antibody staining of intracellular markers

In addition to staining for extracellular markers, some cells were further stained for intracellular markers. For these studies, cells were first stained for extracellular makers as described above before being fixed and permeabilised using FoxP3/Transcription Factor Buffer Staining Set (eBioscience, USA). Next, samples were stained for various cytokines by incubation in a cocktail of fluorophore-conjugated antibodies for 20 min at room

temperature in the dark (see below). Flow cytometric analysis of samples was carried out as described under section 2.8.2 above.

2.8.4 Antibodies used

Antibodies used in sections 2.8.2 and 2.8.3 are as follows,

Table 2.1: Panel of antibodies used for flow cytometry

Antigen	Tag	Company
CD11b	PacB	eBioscience, USA
CD206	PE	BioLegend, USA
CD3	V500	BD Bioscience, USA
CD3	APC	BioLegend, USA
CD4	FITC	BioLegend, USA
CD4	BV605	BioLegend, USA
CD45	APC-Cy7	BioLegend, USA
CD45	AF-700	BioLegend, USA
CD8	BV785	BioLegend, USA
F4/80	APC	BioLegend, USA
F4/80	BV685	BioLegend, USA
IFN-γ	FITC	BioLegend, USA
IL-17	PE-Cy7	BioLegend USA
IL-18R	PE	eBioscience, USA
Ly6G	PE-Cy7	BioLegend, USA

2.8.5 Gating Strategy

A typical flow cytometry gating strategy is shown in Figure 2.1. Specifically, cell debris was first excluded by analysis of side scatter area (SSC-A) vs forward scatter area (FSC-A). "Singlets" were then selected by analysis of forward scatter height (FSC-H) by FSC-A. Live cells vs dead cells were differentiated by analysis of either 7AAD or Aqua Dead Cell staining vs FSC-A. Following this, the total leukocyte population was determined by positive events for CD45 staining. Macrophages were determined by CD45+CD11b+F4/80+ events. Neutrophils were determined by CD45+CD11b+Ly6G+ events. T cells were determined by CD45+CD3+ events which were further divided into either CD4+ (CD4+CD8-) or CD8+ (CD4-CD8+) T cells . IL-17 or IFN-γ expression by leukocytes were determined by positive events for IL-17 or IFN-γ staining respectively.
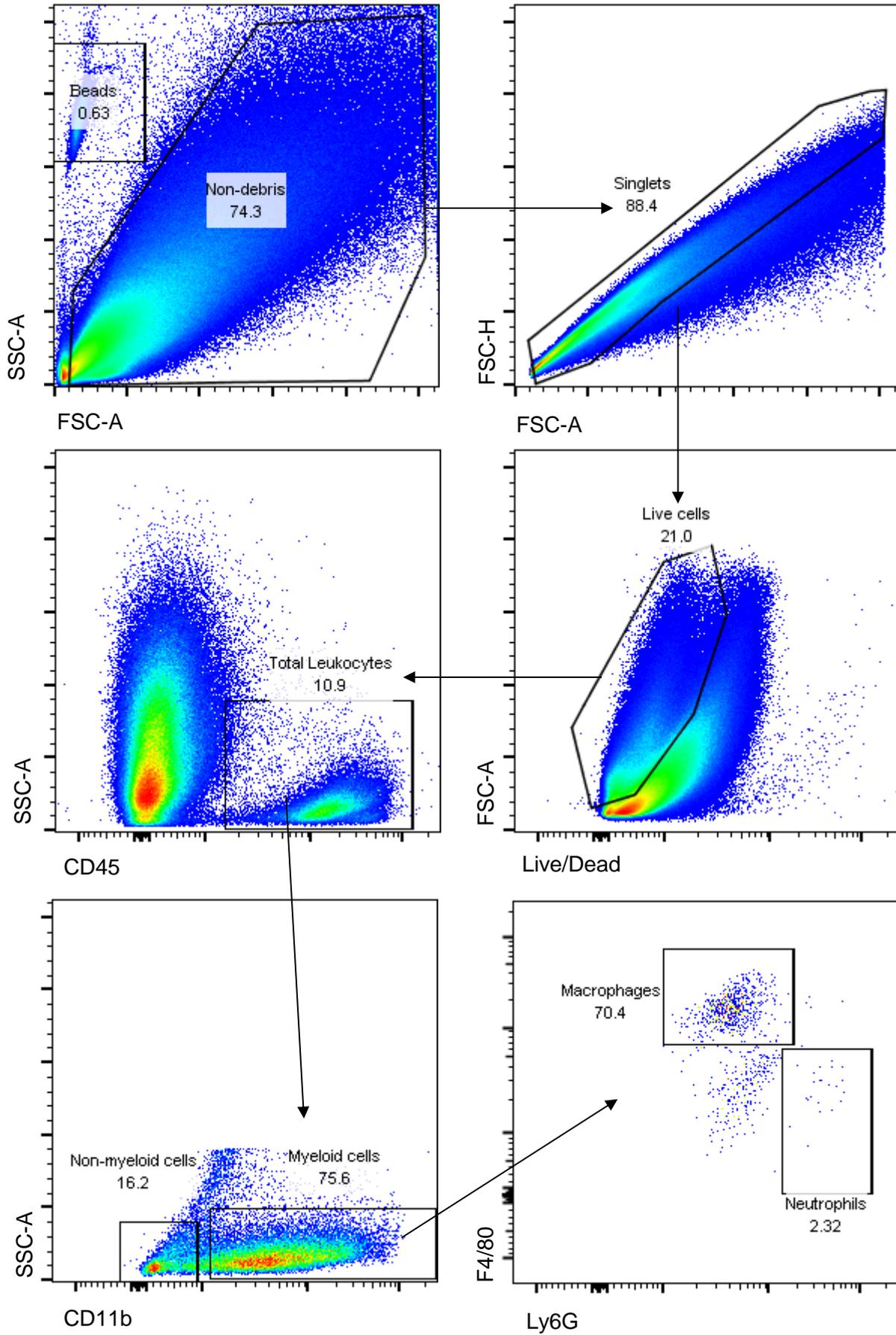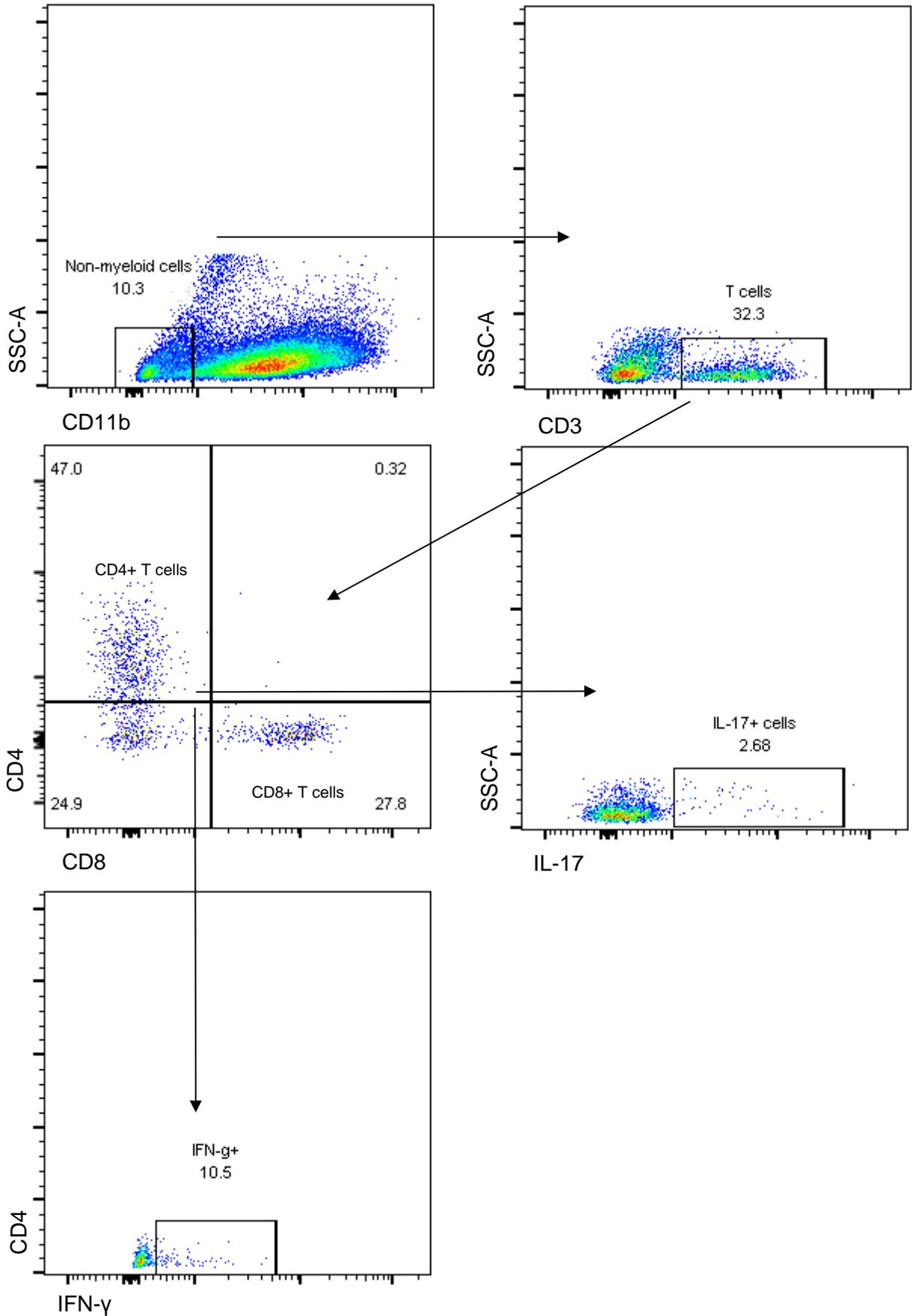
Figure 2.1: Example of gating strategy used as described in section 2.8.5.

2.9 Measurement of plasma antibodies

After the end of the treatment, mice were killed as described in section 2.8.1 above. Blood was collected from the inferior vena cava in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Plasma was collected by centrifugation of blood samples at 10,000 rpm for 10 min at 4°C. Antibody isotopes (IgG1, IgG2a, IgG2b, IgG3, IgA, IgE and IgM) were quantitatively measured using a ProcartaPlex Mouse Antibody Isotyping panel (eBioscience, USA) according to manufacturer's instructions. Briefly, plasma samples were first diluted 1:10,000 in sample diluent. Samples were then loaded in duplicates onto a 96-well plate and incubated with antibody-coated magnetic beads for 60 min at room temperature. After incubation, plates were then secured to a Hand-Held Magnetic Plate Washer and beads were allowed to settle to the bottom of the wells. Plates were washed an additional three time prior to addition of 25 µl of detection antibody to each well. After a further incubation for 30 min at room temperature in the dark, plates were washed and beads resuspended in 120 µl of reading buffer. Plates were then analysed on Bio-Plex® MAGPIX® Multi-Plex Reader (BioRad, USA). Data were analysed using Procartaplex Analyst (eBioscience, USA).

2.10 Cell culture

Immortalised bone marrow-derived macrophages (BMDMs) (kind gift from A. Mansell, Hudson Institute of Medical Research) originally harvested from C57BL/6J mice were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). An immortalised mouse bone marrow-derived macrophage cell line that has been stably transduced with mCerulean tagged ASC was also cultured in DMEM with 10% FBS. The immortalised human monocytic cell line, THP-1 (ATTC, USA), was cultured in high glucose RPMI 1640 medium supplemented with 10% FBS. Wild-type BMDMs and mCerulean tagged BMDMs were passaged every three days at a 1:10 ratio in DMEM + 10% FBS in each flask. THP-1 cells were passaged every three days to contain

1×10^5 cells in each flask. For the extraction of RNA, both wild-type BMDMs and THP-1 cells were seeded at a density of 500,000 cells per well in 6-well plates. THP-1 were first treated with 100 nM of phorbol 12,13-dibutyrate (PDBu) (Sigma, USA) for 24 h to allow differentiation into macrophages. Wild-type BMDMs were allowed at least 12 h to adhere to the plate surface. For the visualisation of ASC specks in BMDMs expressing mCerulean tagged ASC, cells were seeded at a density of 25,000 cells/100 µl per well in Nunc™ Lab-Tek™ 8 well chamber slides for 24 h in DMEM + 10% FBS.

2.10.1 Cell culture treatments

Initial priming of the inflammasome was achieved in all cell types by incubation in 1 µg/ml lipopolysaccharides (LPS) (Sigma) for 4 h. Assembly and activation of the inflammasome was achieved by further incubation in 3 mM adenosine 5′-triphosphate (ATP) (Sigma) for 30 min. To assess the effects of nitric oxide (NO) donors on inflammasome priming/activation, SPER-NO (Cayman Chemical, USA) and SIN-1 (Cayman Chemical, USA) were incubated in conjunction with LPS treatment for 4 h at 250 (or 100, 25, 10) µM and 500 µM respectively. Similarly, to determine the mechanism of NO inhibition on the inflammasome, cyclic GMP inhibitor, ODQ (Cayman Chemical, USA) and NO scavengers, hydroxocobalamin hydrochloride (HC) (Sigma, USA) and carboxy-PTIO (PTIO) (Cayman Chemical, USA) were incubated in conjunction with LPS and SPER-NO for 4 h at a concentration of 10 µM, 200 µM and 100 µM respectively. TLR2, TLR7 and TLR9 activation was achieved using lipoteichoic acid (LTA) (Invivogen, USA) 1 µg/ml for 4 h, imiquimod (Invivogen, USA) 20 µg/ml for 4 h, and CpG ODN (Invivogen, USA) 2 µM for 4 h respectively. The effect of NO on inflammasome priming by activation of these TLRs was determined by incubation of SPER-NO as previously described. To determine if NO mediated its effects on inflammasome priming by inhibition of NF-κB, an NBD (NEMO-binding domain) blocking peptide (Enzo Life Sciences, USA) was incubated for 4 h at 25 µM in conjunction with LPS and SPER-NO treatment as previously described. Furthermore, activation of NF-κB was achieved by using phorbol 12-myristate 13-acetate (PMA) at

concentrations of 1 nM, 10 nM, and 100 nM for 4 h to determine the effect of NF-κB activity on pro-IL-1β expression.

2.10.2 Immunohistochemistry for ASC specking

Twenty four hours after seeding of mCerulean tagged ASC BMDMs in Nunc™ Lab-Tek™ 8 well chamber slides, cells were treated with LPS, ATP and SPER-NO as outlined previously. After the treatment period, cells were washed with phosphate-buffered saline (PBS) (0.01 M) and fixed in 4% paraformaldehyde (PFA) for 15 min. Cells were then washed three times for 15 min with PBS. After physical removal of the plastic walls of the chamber slide system, cover slips were mounted onto microscope slides with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, USA). Slides were viewed and photographed on a Nikon Upright inverted confocal fluorescence microscope (Nikon D-eclipse C1) at 20x magnification.

2.11 Enzyme-linked Immunosorbent Assay for IL-1β and IL-18 levels in cell medium and plasma

Plasma from animals were collected as previously described in section 2.9. Cell medium was collected after the various treatment period and placed in microcentrifuge tubes, snap frozen in liquid N_2 and stored at -80°C. Prior to conduction of the assays, cell medium was centrifuged at 1500 RPM for 5 min to pellet and remove any debris or dead cells. Colorimetric ELISA kits for mouse IL-1β and IL-18 were purchased from Elisakit.com (Australia). The assays were performed according to manufacturer's instructions in 96 well microtiter plates. Briefly, a concentration range of serially diluted standards (supplied by the manufacturer) and samples were loaded directly into the wells of microtiter plates in duplicate and incubated at room temperature for 2 h. Wells were washed with washing buffer prior to addition of a secondary detection antibody consisting of anti-mouse IL-1β or IL-18 conjugated to biotin and incubated for 2 h. Wells were then washed and streptavidin-HRP was added and incubated for 20 min at room temperature. Following this, all wells

were washed and a substrate containing hydrogen peroxide and tetramethyl benzidine was added and incubated for 15 min or prior to overdevelopment of the plate. Reactions were stopped with 0.5M of H_2SO_4 and the absorbance was measured at 250 nm using a microplate reader (Versa max Tunable Microplate Reader; Biostrategy, Australia). The amount of IL-1β or IL-18 in samples was estimated by comparing the optical density (OD) values of the samples with the OD values of the standards.

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

Some kidney halves (composed for renal cortex and medulla) were fixed in 10% formalin, embedded in paraffin and sectioned (5 μm). Section were then deparaffinised, rehydrated and stained with 0.1% Picrosirius red solution. Imaging was performed using a bright-field microscope at x20 or x40 magnification (Olympus, Japan). For some experiments, a polarised filter was used to visualise collagen fibres at x20 magnification (Olympus, Japan). To determine collagen content in bright-field images, the percentage area stained by Picrosirius red in six randomly selected fields-of-view was quantified by a blinded investigator using ImageJ software (National Institute of Health, USA). Random fields of view were determined by dividing the kidney section into six evenly spaced areas after which the investigator randomly selected an area within these six evenly spaced areas to magnify at x20 making sure to avoid brightly stained blood vessels. To determine collagen content in polarised images, the percentage area of collagen fibres in 14 randomly selected fields-of-view was quantified by a blinded investigator using Image J software (National Institute of Health, USA). To determine glomerular cross sectional area, 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.

2.13 RNA extraction, Reverse Transcription cDNA conversion, and Real-time PCR

RNA was extracted from kidneys, BMDM and THP-1 cells using the RNeasy Mini Kit (Qiagen, USA). Kidneys were manually disrupted by repeated mincing with fine scissors and then sonicated in a lysis buffer containing 1% β-mercaptoethanol. WT BMDMs and THP-1 cells were washed in PBS, then scraped and transferred into a microcentrifuge tube containing lysis buffer with 1% β-mercaptoethanol. For kidneys, the resulting lysate was centrifuged at 14,000 RPM for 5 min to remove debris. The supernatant was then loaded into a spin column containing an RNA-binding silica membrane. The RNA was washed with an ethanol containing buffer and then eluted from the column with 30 µl of RNAse-free water. The concentration and purity of the RNA was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA) or Qiaxpert System (Qiagen, USA) by measuring absorbance at 230, 260, and 280 nm. Samples were considered free of protein and phenol contamination and suitable for PCR analysis when the 260/280 and 260/230 ratios were ≥ 2.

1-2 µg of RNA from each sample was reversed transcribed to cDNA using a commercially available kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, USA). Briefly, this involved mixing and incubating the provided reagents of RT Buffer, RT Random Primers, dNTP mix, Multiscribe® Reverse Transcriptase and RNase Inhibitor along with RNA samples using the following thermal cycling parameters; initial step of 25 °C for 10 min; 37°C for 2 h followed by 85°C for 5 min. cDNA samples were then stored at -20°C for further analysis. 10-100 ng of cDNA was used as a template in real-time PCR to analyse mRNA expression of several target and housekeeping genes. Briefly, cDNA was added to the wells of a 96 well plate along with a master mix containing Taqman Universal PCR master mix (Applied Biosystems, Australia) and pre-designed primers for NLRP3, ASC, pro-caspase-1, pro-IL-1β, pro-IL-18, ICAM-1, VCAM-1, CCL5, CCL2, IL-6, IFN-γ, COL1A1, COL3A1, COL4A1, COL5A1, osteopontin, TNFα, IL-17A, vimentin, and the housekeeping gene, GAPDH. Real-time PCR was performed on a Bio-Rad CFX96 Real Time Detection System (Bio-Rad Laboratories, Australia) using the following thermal-cycling parameters;

an initial step of 50°C for 2 min; 10 min at 95°C; 40 cycles of 95°C for 15 sec followed by 60°C for 1 min. Fluorescence was measured at the end of each cycle. Relative expression levels of mRNA were determined using the comparative CT method [1]. The cycle at which fluorescence reached a pre-determined threshold (100 RFU) was defined as the CT value. The ΔCT value was calculated by subtracting the CT value of the housekeeping gene from the target gene. The ΔΔCT value was calculated by subtracting the average ΔCT value of the control group from the treated groups. Fold-changes in gene expression were determined according to the equation:

$$Relative\ mRNA\ expression = 2^{-\Delta\Delta CT}$$

2.14 Statistics

All data is presented as mean ± standard error of the mean (S.E.M.). BP measurements were first analysed using a two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons correction. All other parameters was measured using either a Student's unpaired t-test or a one-way independent measures ANOVA followed by Newman-Keuls multiple comparisons test. Note; post hoc tests were only performed when the F value from the ANOVA was < 0.05. The alpha value was set at 0.05. All statistical tests were conducted using GraphPad Prism v6.0 (Graphpad Software Inc., USA).

References

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Chapter 3:

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

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

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ABSTRACT

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, deoxycorticosterone 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.

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 multimeric 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 25–30 g, 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% O_2). 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-18R, IL-18RAcP, 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].

85

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. Picrosirius 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% Picrosirius red solution. Imaging was performed using a bright-field microscope at ×20 magnification. To determine collagen content, the percentage of area stained by Picrosirius 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 ethylenediaminetetraacetic 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. Picrosirius 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
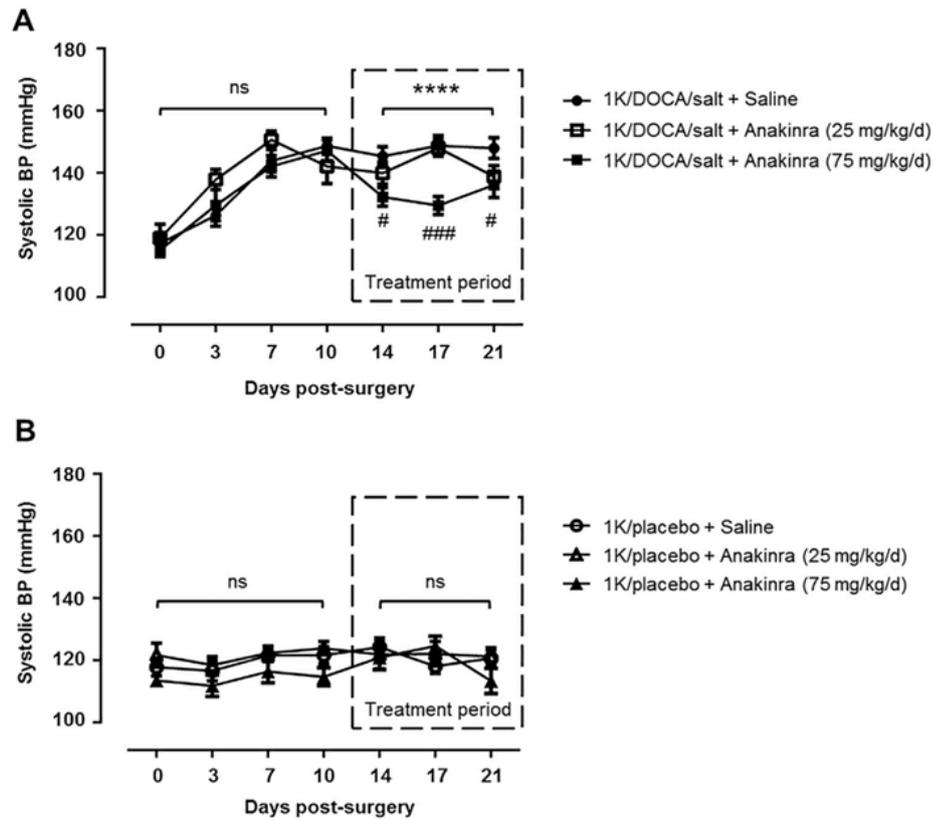
Fig. 1. Effect of anakinra on systolic BP in 1K/DOCA/salt (A) and 1K/placebo-treated (B) mice. Blood pressure was measured via tail-cuff plethysmography. 10 days after surgery, mice were further treated with anakinra (75 or 25 mg/kg/d, i.p.) or vehicle (0.9% saline). Data are expressed as mean ± SEM (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
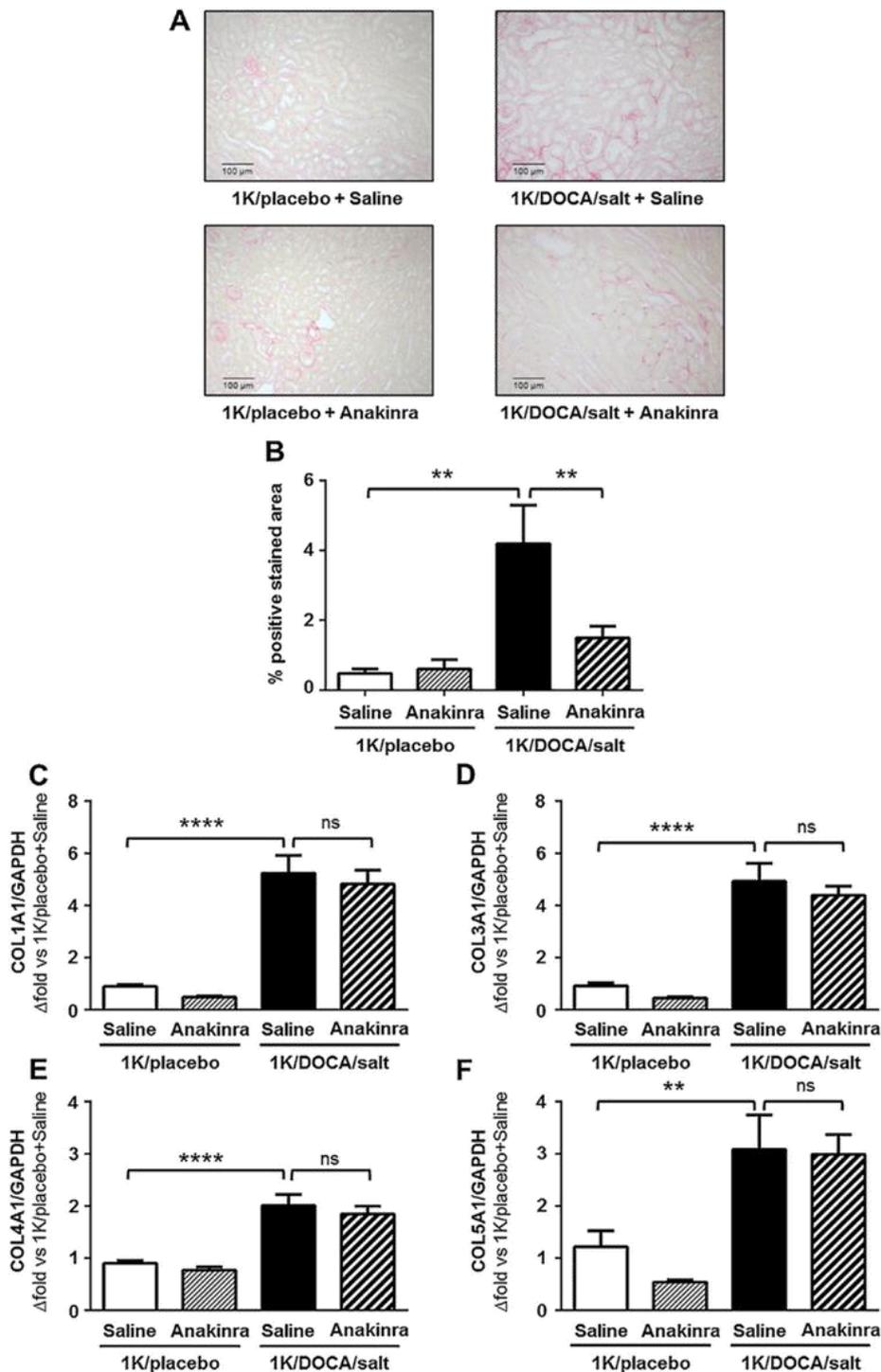
Fig. 2. Effect of anakinra on collagen in the kidneys of 1K/DOCA/salt and 1K/placebo treated animals. (A) Representative image (×20 magnification) and (B) quantified group data of Picrosirius red stained kidney sections. mRNA expression of (C) collagen type 1 alpha 1, (D) collagen type 3 alpha 1, (E) collagen type 4 alpha 1, and (F) collagen type 5 alpha 1. Data are expressed as mean ± SEM (n = 6-8 per group). **$P < 0.01$, ****$P < 0.0001$ for two-way ANOVA followed by Newman-Keuls multiple comparisons test.

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
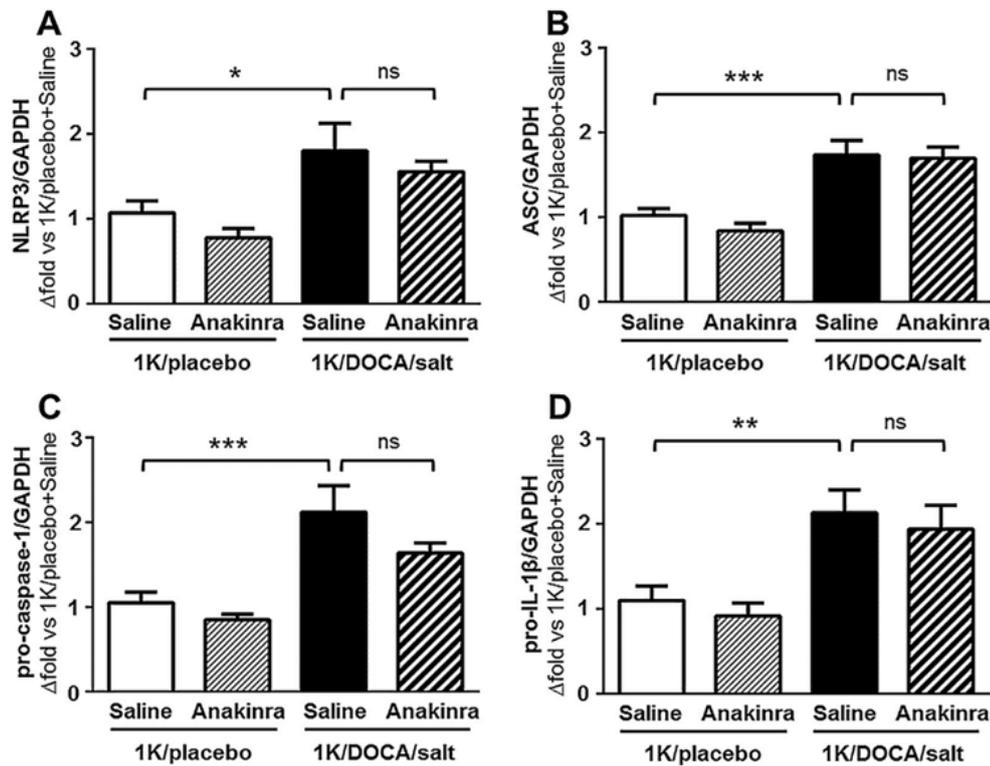
Fig. 3. Effect of anakinra on renal expression of inflammasome subunits; NLRP3 (A), ASC (B), pro-caspase-1 (C) and derived cytokine pro-IL-1β (D). Messenger RNA expression was measured with real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data expressed as mean ± SEM (n = 7–9 per group) *P < 0.05, **P < 0.01, ***P < 0.001 for two-way ANOVA followed by Newman-Keuls multiple comparisons test.

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^+/H_2O 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-18RAcP) 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
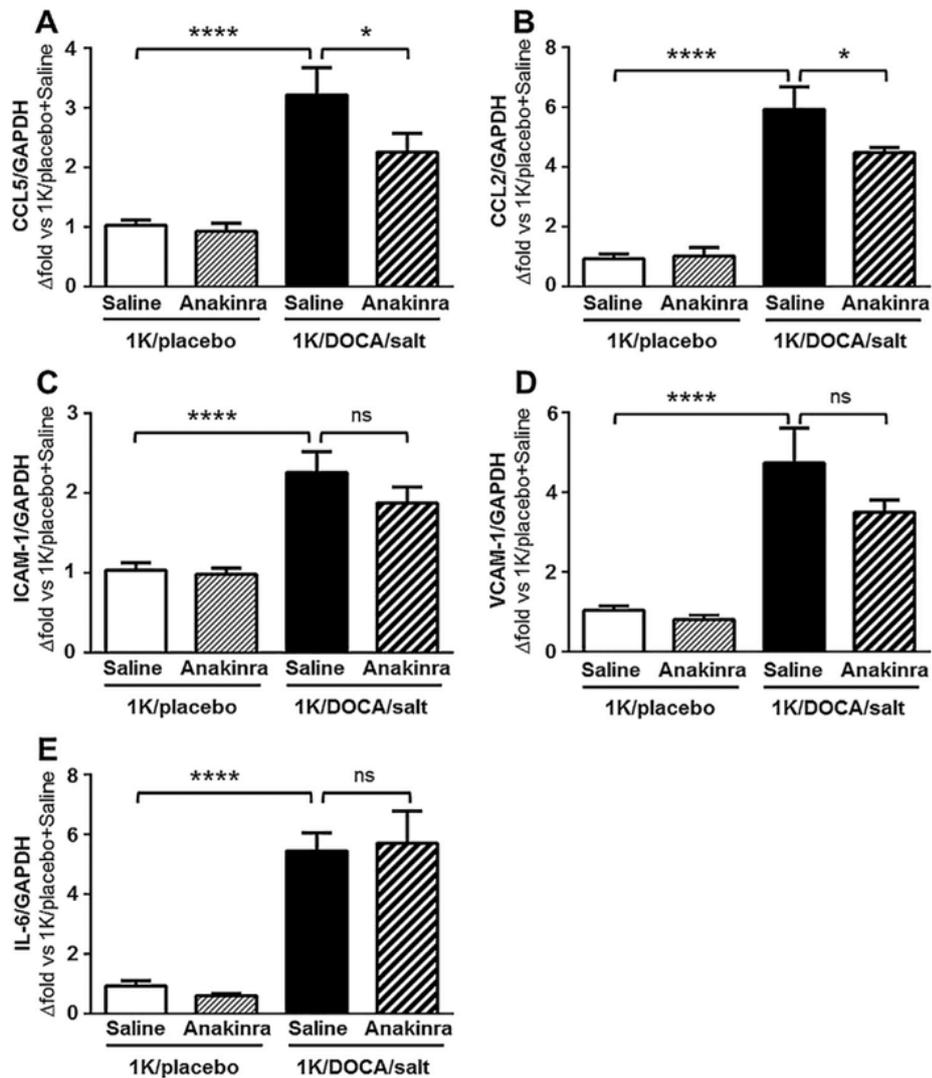
Fig. 4. Effect of anakinra on renal expression of chemokines CCL5 (A) and CCL2 (B); adhesion molecules ICAM-1 (C) and VCAM-1 (D) and the pro-inflammatory cytokine IL-6 (E). Messenger RNA expression was measured with real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data expressed as mean ± SEM (n = 7–9 per group) *P < 0.05, ****P < 0.0001 for two-way ANOVA followed by Newman-Keuls multiple comparisons test.

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 vasore-laxation responses in these animals. Hence, these findings suggest that impaired vasodilator function has only a minor (if any) contri-bution 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 func-tion.

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 com-pared to that of downstream processes such as collagen deposition and elevated BP, such that these latter parameters remained atten-uated 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 anti-bodies could be acting to neutralise the effects of the protein. Regardless, the previous discussion highlights an important lim-itation 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.
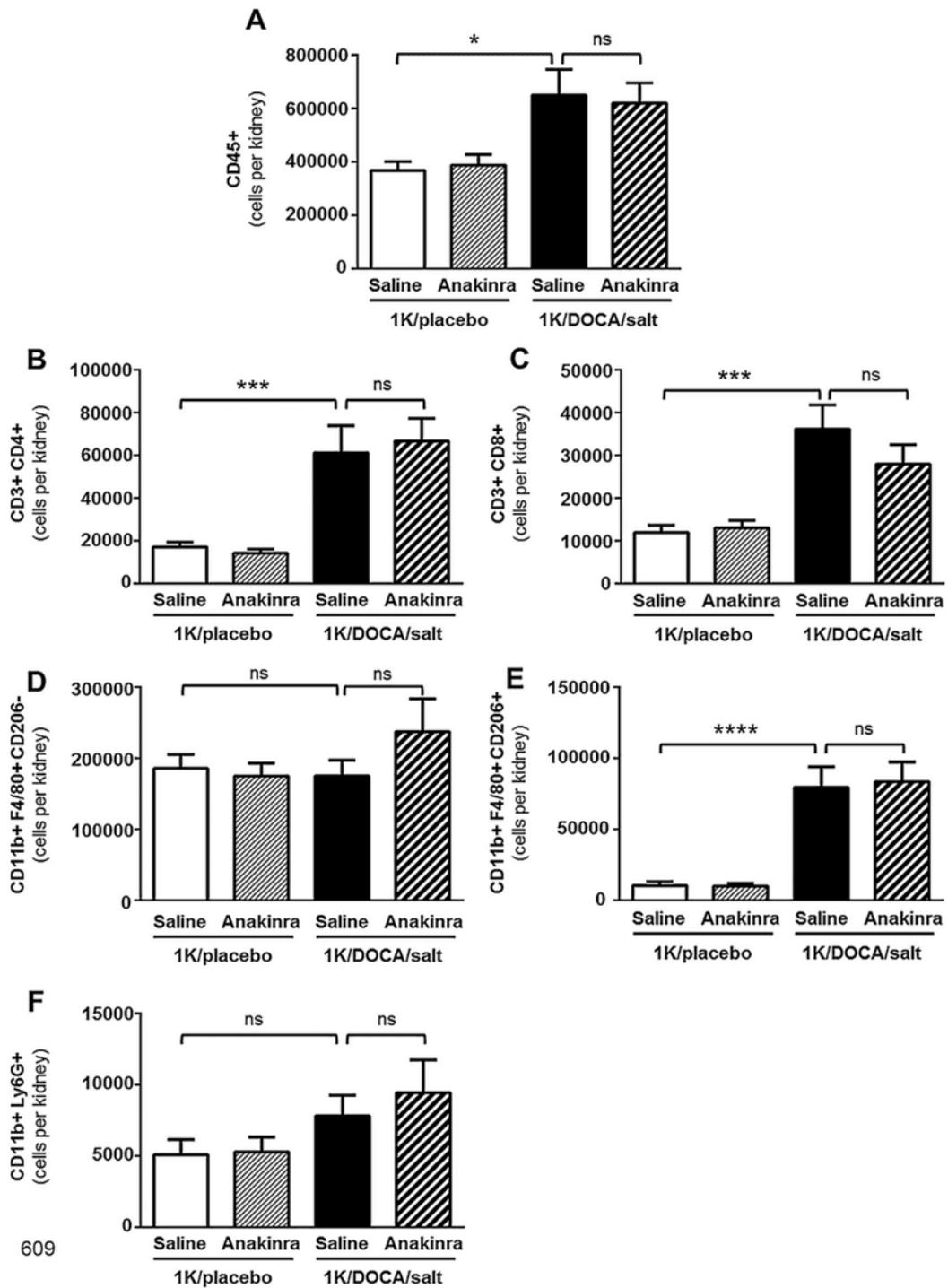
Fig. 5. Effect of anakinra on leukocyte infiltration; Total leukocytes (A), T helper cells (B), cytotoxic T cells (C), "M1" macrophages (D), "M2" macrophages (E) and neutrophils (F) in the kidneys of 1K/DOCA/salt and 1K/placebo-treated mice. Leukocyte infiltration was quantified by flow cytometric analysis. 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-

91
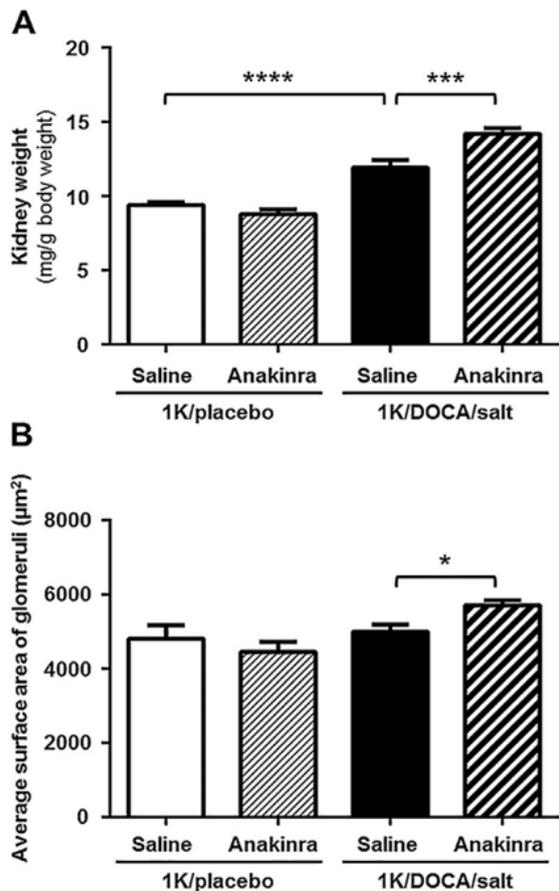
Fig. 6. Effect of anakinra on right kidney weight and glomerular surface area in 1K/placebo and 1K/DOCA/salt treated mice. Kidney weight was normalised to animal body weight (n = 7–9 per group). Glomerular surface area was determined using analysis of Picrosirius red stained sections of the kidney. Data are expressed as mean ± SEM (n = 7-9 per group). *P < 0.05, ***P < 0.001, ****P < 0.0001 for two-way ANOVA followed by Newman-Keuls multiple comparisons test.

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, fur-

ther 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

Acknowledgement

Appendix A.

Previous presentations

ASCEPT Annual Scientific Meeting 2013: Poster Presentation.
State of the Heart 2014 Annual Meeting of ISCP, AAS and HBPRCA: Oral Presentation.
European Congress of Immunology Vienna 2015: Poster Presentation.
HBPRCA Annual Scientific Meeting 2015: Oral Presentation.

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.

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92

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Supplementary Figure 1
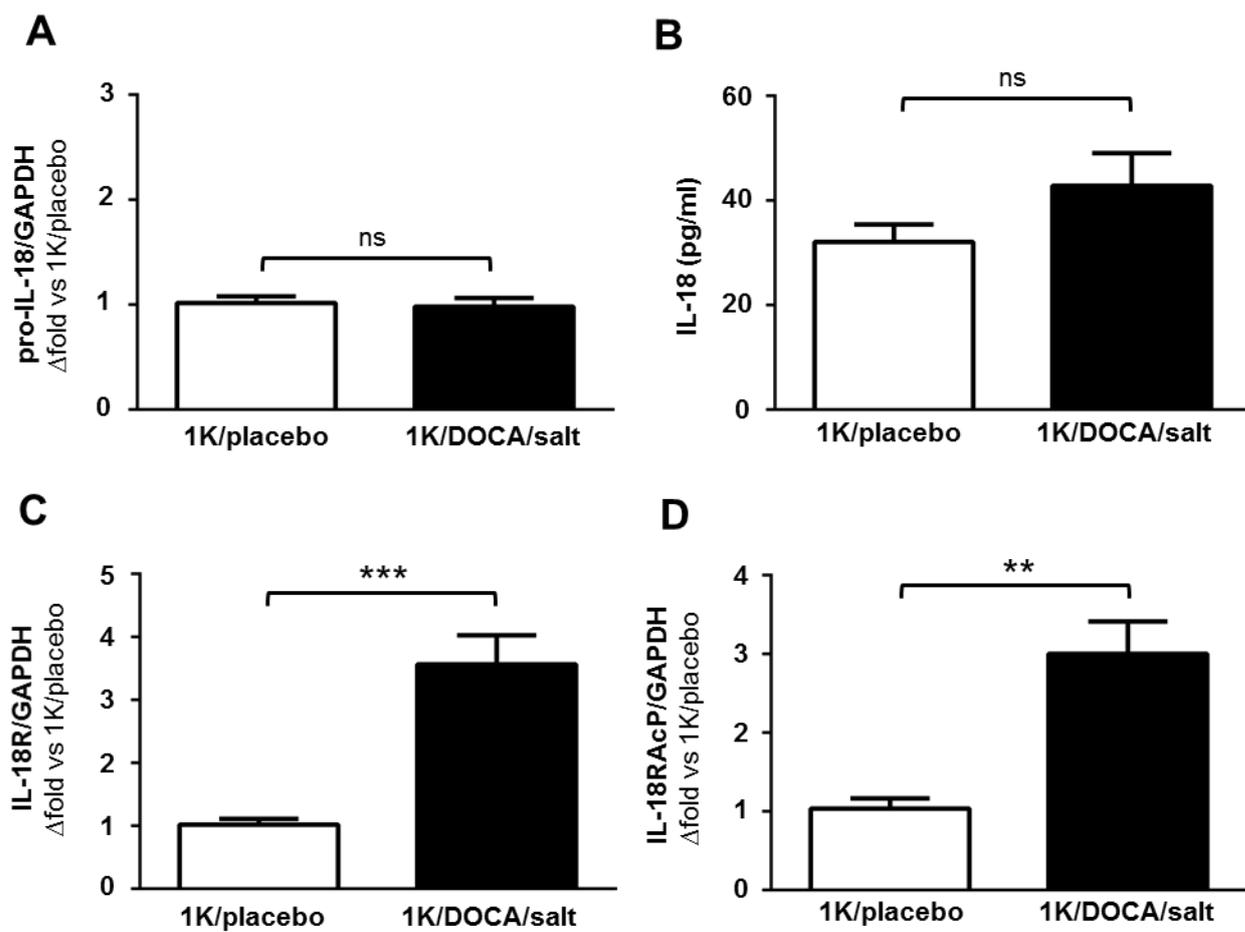
Suppl Fig 1. Effect of anakinra on IL-18 cytokine (A & B) and receptor expression (C & D). Messenger RNA expression was measured with real-time PCR and quantified using the comparative CT method with GAPDH used as the house-keeping gene. Circulating IL-18 was measured using an ELISA. Data are expressed as mean ± SEM (n=6-11 per group). **P<0.01, ***P<0.001 for two-tailed t-test.

Supplementary Figure 2
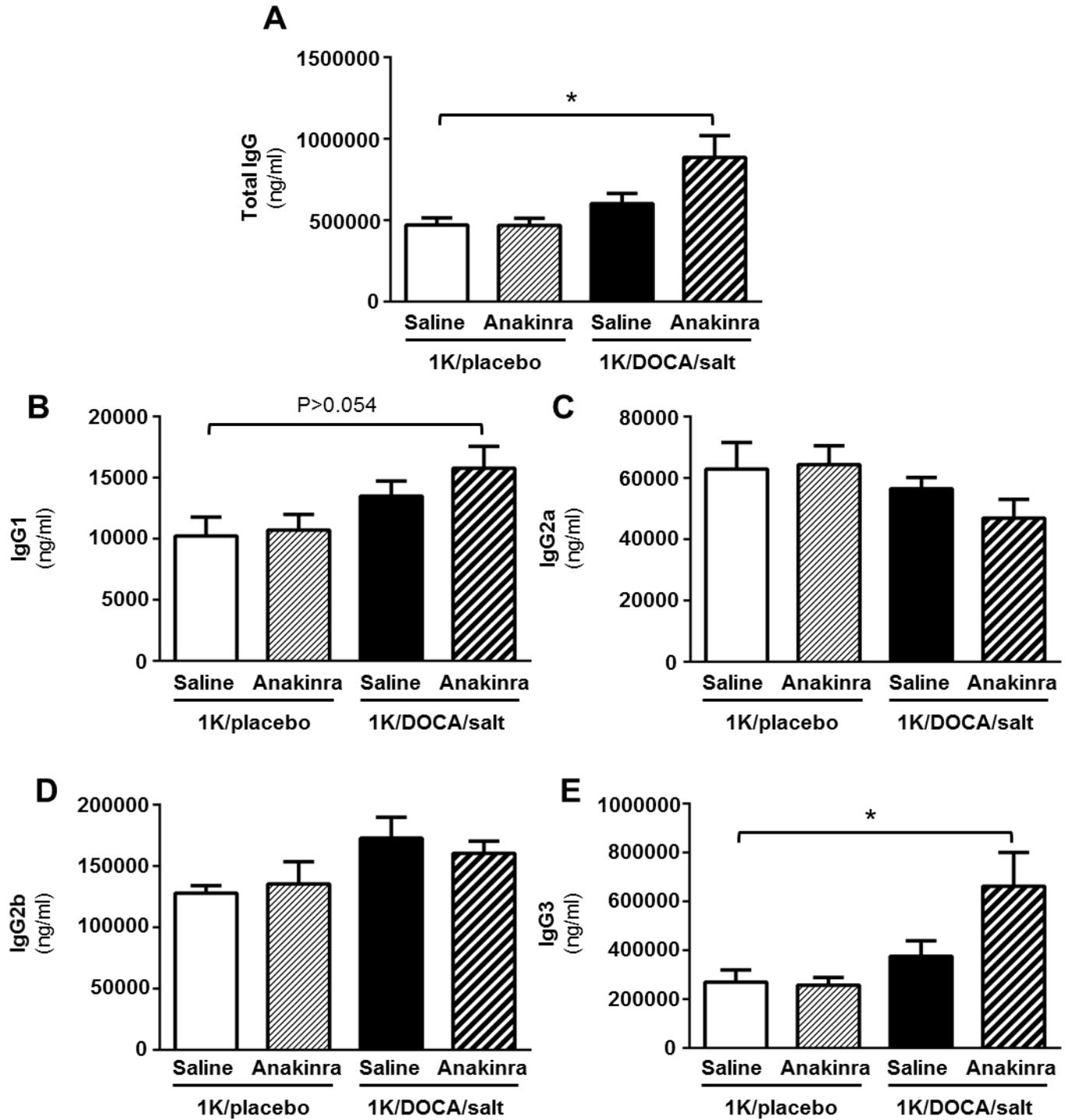
Suppl Fig 2. Effect of anakinra on circulating levels of IgG antibodies in 1K/placebo and 1K/DOCA/salt treated mice. A multiplex immunoassay and Procartaplex™ Analyse 1.0 software were used to determine plasma concentrations of total IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgG3 (E). Data are expressed as mean ±SEM (n=7-9 per group). *$P<0.05$ for one-way ANOVA followed by Newman-Keuls multiple comparisons test.
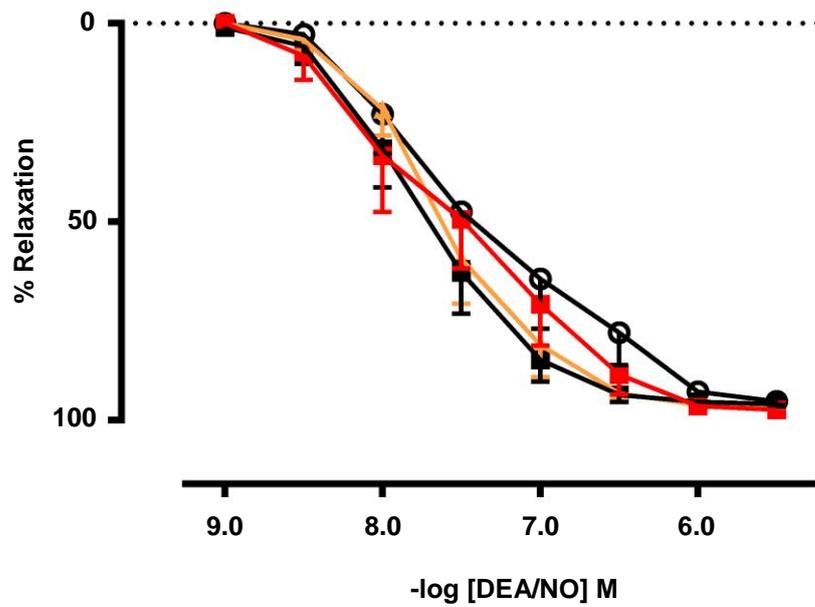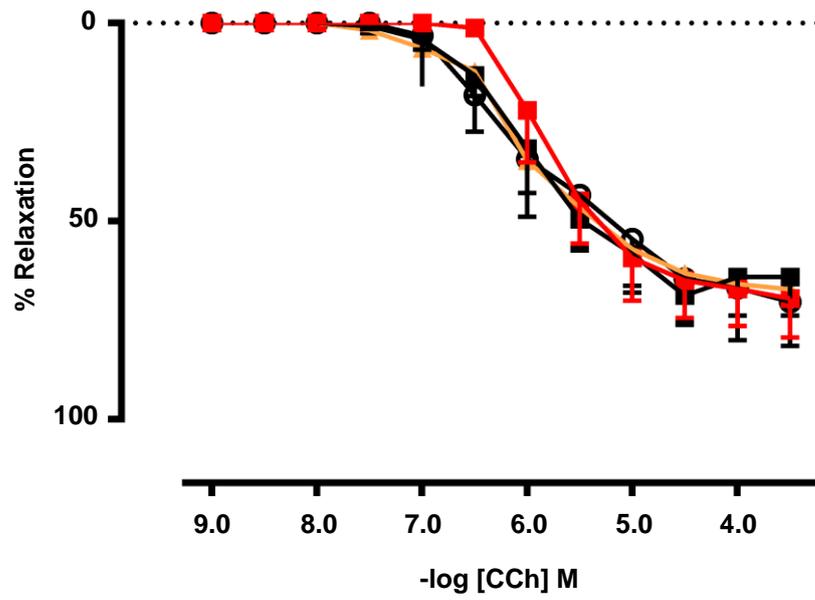
Supplementary Figure 3

Suppl Fig 3. Effect of anakinra on endothelial dysfunction in mesenteric arterial vessels. Data are expressed as mean ±SEM (n=6-8 per group)

Erratum (in response to Examiner's comment)

1. Abstract should make reference to the fact "systolic BP was measured by tail cuff plethysmography"

2. Reference 9 in the bibliography should read as "Abbate, A., Salloum, F.N., Vecile, E., Das, A., Hoke, N.N., Straino, S., et al. (2008). Anakinra, a recombinant human interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. Circulation 117: 2670–2683."

Chapter 4:

Inhibition of IL-18 signalling affords protection against hypertension and renal damage in mice

Abstract

Background: Interleukin-18 (IL-18) is a cytokine released downstream of inflammasome activation that promotes pro-inflammatory T_H1 immune responses. Clinical studies have shown that levels of IL-18 are elevated in hypertensive patients. However, whether IL-18 plays a casual role in hypertension is unknown.

Methods: Hypertension was induced in male wild-type and IL-18$^{-/-}$ mice by uninephrectomy and treatment with deoxycorticosterone acetate (2.4 mg/d, *s.c.*) and 0.9% NaCl in the drinking water (1K/DOCA/salt). Control mice received uninephrectomy, a placebo pellet and normal drinking water (1K/placebo). Systolic blood pressure (BP) was measured by tail cuff and radiotelemetry. After 21 days, kidneys were harvested to assess renal damage; expression of IL-18, IL-18R, and the inflammatory markers ICAM-1, VCAM-1, CCL5, and CCL2 by real time PCR; and immune cell numbers, cellular expression of IL-18R and IL-17 production by T cells with flow cytometry.

Results: 1K/DOCA/salt-treated mice displayed elevated BP (140±3 mmHg) compared to 1K/placebo mice (118±2 mmHg; n≥17, P<0.05). IL-18 expression was ~2-fold higher in the kidneys of 1K/DOCA/salt versus 1K/placebo mice (n≥7; P<0.01). IL-18R expression was also elevated by 3-fold (n≥6; P<0.0001) with infiltrating CD4+ T cells representing the major cell type expressing this receptor. Importantly, IL-18$^{-/-}$ mice were profoundly protected from the hypertensive actions (119±6 vs 145±6 mmHg; n=5, P<0.05) and renal damage (n=6, P<0.05) of 1K/DOCA/salt compared to wild-type mice and displayed marked reductions (i.e. >2-fold) in renal expression levels of ICAM-1, VCAM-1 and CCL2 (n≥4, P<0.05). Furthermore, T cells obtained from hypertensive IL-18$^{-/-}$ mice produced less inflammatory IL-17 compared to hypertensive wild-type mice in response to ionomycin and phorbol myristate acetate (PMA) stimulation (n≥6, P<0.05).

Conclusion: Upregulation of IL-18 and its receptor is a major driver in renal inflammation and the development of hypertension following 1K/DOCA/salt-treatment in mice. Hence,

these findings highlight the IL-18 signalling system as a potential target for future anti-hypertensive therapies.

Introduction

Hypertension is now regarded as a low-grade inflammatory disease. Recent evidence suggests that chronic inflammation occurs in key blood pressure (BP)-regulating organs, such as the kidneys and vasculature, with the family of multimeric protein complexes known as inflammasomes emerging as key mediators [1–7]. Inflammasomes regulate the production of the pro-inflammatory cytokines, IL-1β and IL-18, in response to pathogen-associated molecular patterns (PAMPS) or danger-associated molecular patterns (DAMPS). The NLRP3 inflammasome, consisting of the pattern recognition receptor, NLRP3; an adaptor protein, ASC; and pro-capase-1, is the most well-characterised member of the inflammasome family and deficiency of either ASC or NLRP3 protects mice against the BP changes, renal inflammation and fibrosis that normally accompanies experimentally-induced hypertension [4]. More recently, we have shown that blocking the actions of the downstream cytokine, IL-1β, with a human recombinant IL-1 receptor antagonist, anakinra, partially reverses the BP increases seen in hypertension and reduces the degree of renal interstitial fibrosis at least as measured by Picrosirius red staining [7]. Despite this, anakinra did not reduce the expression of inflammatory markers or the accumulation of inflammatory cells into the kidneys suggesting that IL-1β may not play a significant role in inflammation of the kidneys seen during the development of hypertension. Thus, these findings raise the question, what is the relative contribution of IL-18 - the other inflammasome-regulated cytokine - to renal inflammation and elevated BP during the development of hypertension?

Like IL-1β, IL-18 is a member of the IL-1 cytokine superfamily [8]. Hence, it is generally considered to be pro-inflammatory in nature. IL-18 mediates its actions through activation of its receptor, the IL-18 Type 1 receptor (IL-18R1) [9]. Complete activation of this pathway also requires the recruitment of a homologous, yet unique accessory protein or subunit known as the IL-18 receptor accessory protein (IL-18RAP) [10]. Overstimulation of the system is controlled by the presence of an endogenous inhibitor known as the IL-18 binding

protein (IL-18BP). IL-18BP, like IL-18, is constitutively expressed and is able to bind circulating IL-18, which prevents binding to its receptor thus dampening the system [11].

Clinical studies have shown that hypertensive patients have increased circulating levels of IL-18. Indeed, in a meta-analysis conducted by Rabkin (2009), four out of six studies showed that IL-18 concentrations were higher in the blood of hypertensive patients compared to normotensive patients. However, to date no studies have directly examined whether a cause-effect relationship exists between elevated IL-18 production and the development of renal inflammation and hypertension. Therefore, in the present study we aimed to determine whether the IL-18 system was upregulated in the kidneys during one kidney/deoxycorticosterone acetate/salt-dependent hypertension in mice. We also sought to establish whether mice that are genetically deficient in IL-18 are protected from the BP increases, renal inflammation and damage that normally accompanies this hypertensive model.

Methods

Animals

Male wild type, IL-18$^{-/-}$, IL-18R$^{-/-}$ and RAG1$^{-/-}$ mice (on a C57BL/6J background) [13] of age 10-12 weeks and weighing 25-30 g were used in this study. All strains of mice were bred and housed at the Monash Animal Research Laboratories (ARL; Monash University, Australia) or La Trobe Animal Research and Teaching Facility (LARTF). All 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 Monash Animal Research Precinct Animal Ethics Committee (Project number: MARP/2015/034).

Adoptive transfer of purified T cells

T cells were isolated from total splenocytes using a commercially available cell-specific isolation kit (Miltenyl Biotech, USA) and negative magnetic sorting (AutoMACS). Immediately after cell isolation, 2×10^7 cells were resuspended in sterile PBS, filtered through a 70 μm filter and injected intravenously via the tail vein into RAG1$^{-/-}$ mice.

Induction of hypertension

Mice were placed under general anaesthesia using a flow regulated isoflurane machine (0.4 L/min, 2.5% in O$_2$). 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 drinking water with 0.9% saline (1K/DOCA/salt). Normotensive mice were also uninephrectomised but received a placebo pellet and were maintained on normal drinking water (1K/placebo).

Blood pressure measurements

BP was measured either via tail cuff plethysmography or radiotelemetry. Mean arterial pressure (MAP) and heart rate were also measured by radiotelemetry. Tail cuff plethysmography was conducted 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 weekly thereafter. For radiotelemetry, a telemeter probe (Model TA11PA-C10, Data Sciences International, USA) was surgically implanted in mice. Briefly, this involved placing mice under general anaesthesia as above after which a midline incision was made. A subcutaneous pouch was made along the right flank of the animal into which the telemeter probe was implanted. Fine forceps were used to tease aside surrounding tissue to reveal the left carotid artery. The left carotid artery was gently lifted by placing curved forceps beneath it. Sterile 5-0 sutures were used to make three knots along the artery for insertion and positioning of the catheter tip. One suture was tied rostral to the forceps, a second suture was tied caudal to the forceps; and a loose knot was made with the third suture between the other two sutures. A small incision was then made in the carotid artery rostral to the first suture and opened using fine forceps. Prior to insertion, the telemeter probe was turned on using a magnet and checked using a radio receiver. The probe catheter was then carefully inserted into the incision made in the artery after which the middle and caudal sutures were secured with a double knot. The telemeter probe was then inserted carefully into the subcutaneous pouch made earlier. After repositioning all surrounding tissue, incisions were closed using sterile 6-0 surgical sutures and non-continuous stitches. After allowing 10 days for recovery, baseline measurements were taken for three days by monitoring BP, heart rate, and locomotor activity continuously over a 24 h period in freely moving animals. Following this, mice underwent surgery to induce hypertension as previously outlined with 24 h radiotelemetric monitoring continuing throughout the treatment period.

At the end of the treatment period, mice were killed by isoflurane (Baxter Healthcare, Australia) or CO_2 overdose. The thoracic and abdominal cavities were exposed via a ventral incision. A cannula was inserted into the left ventricle and mice were perfused with 0.2% clexane (400 IU; Sanofi Aventis, Australia) in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) with the aid of a perfusion pump (Cole-Parmer, USA). The right kidney was removed and used for *ex vivo* analysis.

Measurement of mRNA expression levels

RNA was extracted from kidneys 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) or Qiaxpert System (Qiagen, USA). RNA was reverse transcribed to cDNA for use as a template in real-time PCR using a High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA). Real-time PCR was conducted using Taqman® primers and probes for pro-IL-18, IL-18R1, IL-18RAP, IL-18BP, CCL5, CCL2, ICAM-1, VCAM-1, IL-6, collagen type 1 alpha 1 (COL1A1), collagen type 3 alpha 1 (COL3A1), collagen type 5 alpha 1 (COL5A1) and the house keeping gene, GAPDH (Taqman Gene Expression Assays, Applied Biosystems, USA). Real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Australia). Fold-changes in mRNA expression relative to control samples were determined using the comparative CT method [14].

Flow cytometric analysis of leukocytes in the kidney

From some mice, kidneys were removed and enzymatically digested using a cocktail of collagenase enzymes including collagenase type XI (125 U/ml), collagenase type I-S (460 U/ml) and hyaluronidase (60 U/ml) (Sigma-Aldrich, USA) in PBS modified with calcium chloride and magnesium chloride (Sigma-Aldrich, USA). Samples were then passed through a 70 µm cell strainer before being subjected to a Percoll® gradient spin. Cells were

then washed with PBS and stained with an antibody cocktail consisting of anti-mouse CD45 (AF-700; BioLegend, USA), anti-mouse CD3 (APC; BioLegend, USA), anti-mouse CD4 (BV605; BioLegend, USA), anti-mouse CD8 (BV785; BioLegend, USA), anti-mouse F4/80 (BV785; BioLegend, USA), anti-mouse IL-18R (PE; eBioscience, USA),

To measure IL-17 production by immune cells, cells were initially isolated as above, then stimulated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (20 ng/ml) for 5 h in the presence of Golgiplug/Golgistop (BD Biosciences, USA). Following stimulation, cells were stained for extracellular markers as above then permeabilised to allow for intracellular staining of IL-17 using a FoxP3/Transcription Factor Buffer Staining Set (eBioscience, USA) along with a separate antibody cocktail consisting of anti-mouse IL-17 (PE-Cy7; BioLegend, USA). Samples were analysed by flow cytometry using a BD LSR Fortessa™ (BD Bioscience, USA) and FlowJo Software (version 10.1, Tree Star Inc, USA). Cells numbers were normalised using CountBright counting beads (Invitrogen, USA) and expressed as total cells per kidney or as a percentage of the parent cell subset.

Picrosirius red staining for the detection of renal interstitial collagen

Some kidney halves (cut coronally and 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% Picrosirius red solution. Imaging was performed using a bright-field microscope at 20x magnification. To determine collagen content, the percentage area stained by Picrosirius red in six randomly selected fields of view was quantified by a blinded investigator using ImageJ software (National Institute of Health, USA).

Statistical analysis

Data are expressed as mean ± SEM. Data were analysed either by Student's unpaired t-test, one-way ANOVA or two-way Repeated Measures ANOVA followed by Newman-Keuls *post hoc test* or Bonferonni *post hoc* test where appropriate. $P < 0.05$ was considered to be

statistically significant. NB *post hoc* tests were only performed when the F test from the ANOVA indicated statistical significance. Data were graphed and analysed using Graphpad Prism Software v6.04

Results

<u>1K/DOCA/salt-induced hypertension is associated with increased expression of the IL-18 system in the kidneys</u>

Consistent with our previous findings [4,7,15], 1K/DOCA/salt treatment in mice caused a rapid increase in systolic BP such that by day 7 of treatment, systolic BP was approximately 20 mmHg higher than that in the 1K/placebo controls. BP remained elevated at a similar level in the 1K/DOCA/salt-treated mice until the end of the 21 day treatment period (Fig 1A). Real-time PCR revealed that 1K/DOCA/salt hypertension in mice was associated with increased renal mRNA expression of several components of the IL-18 system, including pro-IL-18 (Fig 1B), the Type 1 IL-18 receptor (IL-18R1) (Fig 1C), the IL-18 receptor accessory protein (IL-18RAP) (Fig 1D), and the IL-18 binding protein (IL-18BP) (Fig 1E).

<u>1K/DOCA/salt hypertension is associated with increased accumulation of IL-18R1 expressing leukocytes in the kidneys</u>

Using flow cytometric analysis, we showed that 1K/DOCA/salt-induced hypertension was associated with the accumulation of IL-18R1-expressing leukocytes in the kidneys such that these cells were present in 4-fold higher numbers than in kidneys from normotensive mice (Fig 2A). Further analysis of the immune cell subsets that expressed the IL-18R1 in the kidneys of hypertensive mice revealed that >50% of IL-18R1^{+} cells were T cells, with macrophages making up ~7% of the IL-18R^{+} cell population (Fig 2B). Of the IL-18R1^{+} T cells, the majority were CD4+ T cells (Fig 2B). Based on the above observations we next wanted to determine whether inhibition of IL-18 signalling might afford protection against the elevated BP and renal inflammation that occurs during the development of 1K/DOCA/salt-induced hypertension.

Chapter 4: IL-18 inhibition in hypertension

<u>IL-18 deficiency protects against BP increases in 1K/DOCA/salt-induced hypertension</u>

Consistent with our earlier findings (see Fig 1A), treatment of wild type mice with 1K/DOCA/salt caused systolic BP (as measured by tail cuff) to increase by ~ 20 mmHg by day 7, with this elevated level of BP persisting until the end of the 21 day treatment period. However, 1K/DOCA/salt-induced elevations in BP were markedly blunted in IL-18$^{-/-}$ mice such that systolic BP only rose by ~ 10 mmHg (Fig 3A). Findings from radiotelemetry experiments mirrored those from tail cuff studies with systolic BP in this cohort of IL-18$^{-/-}$ mice only rising to approximately half the level seen in wild type mice following 1K/DOCA/salt treatment (Fig 3B). Interestingly, IL-18$^{-/-}$ mice did not appear to be protected from 1K/DOCA/salt-induced increases in diastolic BP compared to normotensive mice(Fig 3C), nor was there any impact of IL-18-deficiency on heart rate (Fig 3E).

<u>IL-18 deficiency reduces mRNA expression of components of the IL-18 system, inflammatory markers, and pro-inflammatory T cells in the kidneys</u>

Analysis of renal mRNA expression at the end of the 21 day 1K/DOCA/salt treatment regimen revealed that IL-18$^{-/-}$ mice were virtually devoid of pro-IL-18 (Fig 4A), confirming that they were indeed deficient in the gene of interest. This lack of expression of pro-IL-18 in IL-18$^{-/-}$ mice was further associated with reduced levels of IL-18R1 (Fig 4B), but no significant differences in the expression of IL-18RAP (Fig 4C) or IL-18BP (Fig 4D) compared to wild-type mice.

We have previously shown that 1K/DOCA/salt hypertension in mice is associated with upregulation of several inflammatory markers in the kidneys including the adhesion molecules ICAM-1 and VCAM-1; the chemokines CCL5 and CCL2, and the pro-inflammatory cytokine IL-6 (ref). Real-time PCR revealed that expression of ICAM-1 and VCAM-1 in the kidneys following 1K/DOCA/salt was blunted by 60-80%in IL-18$^{-/-}$ compared to wild type mice (Fig 5A & 5B). IL-18$^{-/-}$ mice treated with 1K/DOCA/salt also appeared to

display reduced expression of CCL5, CCL2, and IL-6 compared to wild types (Fig 5C, 5D, & 5E), however these apparent differences did not reach statistical significance.

Adhesion molecules and chemokines are important for the attraction of leukocytes to sites of inflammation. Therefore, we sought to determine whether reduced expression of these molecules in IL-18 deficient mice was reflected by a similar reduction in 1K/DOCA/salt-induced leukocyte accumulation in the kidneys. Flow cytometric analysis of kidney cell suspensions from wild type mice revealed a trend for an increase in total CD45+ leukocyte in the kidneys of hypertensive mice (Fig 6A), as well as similar trends for increases in T cell (Fig 6B) and macrophage populations (Fig 6C). Surprisingly, IL-18 deficiency did not afford protection against leukocyte accumulation, and if anything, the increases in numbers of total leukocytes, T cells and macrophage populations were more robust in IL-18$^{-/-}$ compared to WT mice (Fig 6A-C).

Although IL-18 deficiency had little effect on the overall accumulation of leukocytes in the kidneys, we did find evidence to suggest that less pro-inflammatory T cells accumulated in the kidneys of IL-18$^{-/-}$ mice following 1K/DOCA/salt-treated compared to wild type mice. Specifically, while there was a three-fold increase in the number of IL-17 producing T cells in the kidneys of WT mice following 1K/DOCA/salt-treatment, no such increase was seen in IL-18$^{-/-}$ mice (Fig 6D). In support of the concept that IL-18 acts directly on T cells to promote IL-17 production, we found that it was the IL-18R1$^+$ (and not the IL-18R1$^-$) T cells that were responsible for IL-17 production during 1K/DOCA/salt hypertension (Fig 6E and F).

<u>IL-18 deficiency reduces renal fibrosis in 1K/DOCA/salt-treated mice</u>

Excessive collagen deposition leading to fibrosis and dysfunction can occur as a result of tissue damage and inflammation. Therefore, we investigated whether the reduced degree of renal inflammation observed in IL-18$^{-/-}$ mice compared to WT mice following 1K/DOCA/salt-treatment was reflected by a reduction in renal interstitial collagen

deposition. There was ~60% less interstitial collagen, as visualised by Picrosirius red staining, in the kidneys of 1k/DOCA/salt-treated IL-18^{-/-} mice compared to wild type mice (Fig 7A & 7B). The mRNA expression levels of the four major collagen sub-types expressed in the kidneys, collagen I (COLA1A1) (Fig 7C), collagen 3 (COL3A1) (Fig 7D), collagen 4 (COL4A1) (Fig 7E) and collagen 5 (COL5A1) (Fig 7F) were also reduced in IL-18^{-/-} mice, suggesting that the reduction in collagen protein levels was at least partially due to downregulation of collagen gene activity.

<u>Adoptive transfer of T cells from IL-18R1-deficient mice fails to protect RAG1^{-/-} mice from the development of 1K/DOCA/salt hypertension</u>

Having showing that T cells were the major source of the increased IL-18R1 in the kidneys of 1K/DOCA/salt-treated mice, we next examined whether mice with T cell-specific deficiency of this receptor subunit were protected against the development of hypertension. RAG1^{-/-} mice are normally devoid of T cells in all organs, including the kidneys (Fig 8A). Adoptive transfer of either WT or IL-18R1-deficient T cells into RAG1^{-/-} mice was highly effective at reconstituting T cell populations in the kidneys (Fig 8A) and spleen (data not shown). Importantly, there was no sign of IL-18R1 expression on T cells in RAG1^{-/-} mice that were adoptively transferred with IL-18R1-deficient T cells (Fig 8B). Not surprisingly, RAG1^{-/-} mice that received adoptive transfer of wild type T cells developed hypertension in response to 1K/DOCA/salt treatment (Fig 8C). However surprisingly, RAG1^{-/-} mice that received IL-18R1-deficient T cells developed a similar degree of hypertension (Fig 8). In other words, adoptive transfer of IL-18R1-deficient T cells did not appear to afford protection against the development of 1K/DOCA/salt-induced hypertension.

Discussion

The main novel findings of this study are that: (1) several elements of the IL-18 signalling system including IL-18 itself, as well as levels of the two main IL-18 receptor subunits IL-18R1 and IL-18RAP, are upregulated in the kidneys of mice during the development of 1K/DOCA/salt hypertension; (2) inhibition of IL-18 signalling by knocking out the IL-18 gene not only prevents hypertension but also profoundly attenuates renal inflammation and fibrosis. (3) Mice with T cell specific deletion of IL-18R1 are not protected against the development of hypertension, raising the possibility that other components of the IL-18 signalling system (e.g. IL-18RAP) may be more viable targets for future therapies to treat hypertension and chronic kidney disease.

Consistent with previous studies using the 1K/DOCA/salt-induced hypertension model, we observed an increase in BP by day 10 post-surgery, which was maintained until the end of the treatment period [4,7,15]. The present study further showed that this increase in BP was associated with increased gene expression of several elements of the IL-18 signalling system in the kidneys, including IL-18, its receptor, IL-18R1, and the IL-18 receptor accessory protein, IL-18RAP. Furthermore, flow cytometric analysis revealed that the likely reason for the increase in IL-18R1 expression in the kidneys following 1K/DOCA/salt treatment was that there was an increase in the number of leukocytes expressing this receptor subunit. Further analysis of the specific leukocyte subset that expressed the IL-18R1 revealed that there was an increase in the proportion of IL-18R1+ T cells in the kidneys of hypertensive mice, especially CD4+ T cells. This finding highlights T cells as likely targets for IL-18 in the kidneys during hypertension, and potential mediators of the inflammation that occurs in this organ during the disease. Indeed, T helper (Th) cells, a subset of the CD4$^+$ T cell population (along with T$_{regs}$), are key regulators of adaptive immunity and the associated inflammation through their ability to release pro-inflammatory cytokines such as IFN-γ and IL-17. Previous studies have shown that IL-18, working in concert with IL-12, is a key stimulus for the production of IL-17 and IFN-γ by T cells [16,17].

IFN-γ and IL-17 can act as chemoattractants to facilitate the recruitment of other immune cell types such as monocytes, macrophages, and neutrophils to sites of inflammation [18–20]. IL-18 has also been shown to act directly on other cell types such as endothelial cells and monocytes where it promotes upregulation of adhesion molecules, such as ICAM-1 [21,22]. Alongside IL-17 and IFN-γ, adhesion molecules would be expected to promote inflammation by facilitating the extravasation of inflammatory cell types from the vasculature to the kidneys. Prolonged inflammation of the kidneys eventually leads to fibrosis, impaired functional performance and disruption of the pressure-natriuresis relationship, leading to sodium and water retention, and increased arterial BP [23–27]. Thus, strategies which interfere with renal inflammation should be beneficial for the treatment of hypertension.

Indeed, in this study, we showed that IL-18 gene deficiency affords marked protection against the increased BP seen in 1K/DOCA/salt-induced hypertension. Despite having similar baseline BPs to WT mice, IL-18$^{-/-}$ mice displayed blunted pressor responses to 21 d of 1K/DOCA/salt-treatment. Furthermore, while systolic BP (and MAP) was decreased in IL-18$^{-/-}$ mice, neither diastolic BP nor heart rate were affected. The lack of effect of IL-18-deficiency on diastolic BP suggests the cytokine's role in BP regulation is unlikely to involve a major influence on total peripheral resistance. Rather, systolic BP is largely influenced by cardiac output, which in turn is influenced by heart rate and stroke volume. Hence, the protective actions of IL-18-deficiency on systolic BP without affecting HR is consistent with the concept that IL-18 regulates BP pressure at the level of blood volume regulation by the kidneys. Of note, antihypertensive agents that selectively lower systolic BP, are thought to be of greater benefit than agents which lower all BP parameters due to a phenomenon known as the "J-curve effect" [28]. While it is obviously desirable to lower BP back to normotensive levels (i.e. 120/80 mmHg), aggressive BP lowering resulting marked falls in diastolic BP may actually increase the risk of cardiovascular events due to a reduction in coronary blood flow [28]. Therefore, our observation that IL-18$^{-/-}$ mice have reduced systolic

BP but not diastolic BP could highlight IL-18 blocking therapies as a safer approach for the treatment of hypertension compared to general vasodilator agents.

Expression of the IL-18 signalling system in IL-18 deficient mice was reduced in the kidneys of IL-18$^{-/-}$ mice both in terms of IL-18 itself and also expression of IL-18R1. This suggests a maladaptive positive feedback loop whereby IL-18 production promotes upregulation of the IL-18R. Expression levels of some inflammatory markers that lie downstream of IL-18 were also reduced in IL-18 deficient mice, including the adhesion molecules ICAM-1 and VCAM-1. Interestingly, the reduction in IL-18 signalling, ICAM-1, and VCAM-1 only seemed to impact on the accumulation of a small subset of IL-17 producing T cells. While the reason for this is unclear at present, these IL-17 producing T cells seem to be solely driven by the activity of IL-18. It is thus possible that these activated IL-17 producing T cells are responsible for the inflammation and end organ damage due to hypertension. Indeed, IL-17$^{-/-}$ mice are known to be protected against BP increases, vascular dysfunction and superoxide production in angiotensin II induced hypertension [29]. Therefore, the effects of IL-18 on the production of IL-17 by T cells is likely to be a key driver of inflammation in 1K/DOCA/salt-induced hypertension.

As previously mentioned, interstitial fibrosis of the kidneys is thought to lead to disruption of the pressure-natriuresis relationship, leading to Na$^+$/H$_2$O retention and increased arterial BP [23–27]. In the present study, we showed that IL-18 deficiency reduces collagen deposition in the interstitium of the kidneys during 1K/DOCA/salt-induced hypertension and that this was also associated with a reduction in the gene expression of several key collagen sub-types. This finding is consistent with our previous studies demonstrating that inhibition of inflammasome activation or IL-1β signalling is effective at reducing renal damage in 1K/DOCA/salt-induced hypertension [4,7].

Furthermore, the current study shows that the expression of IL-18BP is increased in 1K/DOCA/salt hypertension. As IL-18BP is an endogenous inhibitor of the activity of IL-18,

it is possible that this upregulation is a compensatory mechanism in response to the increased IL-18 activity seen in 1K/DOCA/salt hypertension. However, it does seem that endogenous inhibition is not sufficient to completely block the detrimental effects of IL-18 in hypertension, with IL-18 gene deficiency being required to see the beneficial effects of IL-18 inhibition in the current study. As gene therapy is unlikely in humans for the treatment of hypertension, this highlights the possible therapeutic option of exogenous delivery of IL-18BP as a treatment for "boosting" existing endogenous inhibitory systems. While such a treatment has not been investigated in the context of cardiovascular diseases, IL-18BP is already in clinical trials for the treatment of Adult-onset Still's disease, a rare auto-inflammatory disease.

It was interesting to note that whereas IL-18 deficiency afforded profound protection against the hypertensive effects 1K/DOCA/salt, IL-18R1 deficiency (albeit only in T cells) did not. One obvious interpretation of these findings is that IL-18 mediated its pro-hypertensive effects in a T cell-independent manner. Hence, it would be interesting in future studies to investigate first if mice that are globally deficient in IL-18R1 are resistant to 1K/DOCA/salt hypertension and then explore which IL-18R1-expressing cell type(s) are responsible for the pro-hypertensive actions of the cytokine. However, it is also worth noting that IL-18R1 has been implicated in both pro- and anti-inflammatory signalling and hence inhibition of IL-18R1 may not be the most effective approach for inhibiting the pro-hypertensive effects of IL-18. IL-37 is a more recently identified member of the IL-1 family and, in contrast to IL-1β and IL-18, has been shown to have anti-inflammatory properties. IL-37 is known to utilise the same receptor subunit as IL-18, the IL-18R1, but upon binding it recruits a different set of accessory proteins, namely IL-1R8 and IL-1R9 to exert anti-inflammatory effects [30,31] (Fig 9). Therefore, this knowledge, along with findings from the current study might suggest that targeting the accessory protein that specifically skews the IL-18 signalling system toward a pro-inflammatory response - IL-18RAP – is a better approach to reducing

inflammation in hypertension. Hence, in future studies it would be interesting to focus on the effect that IL-18RAP deficiency has on the development of hypertension.

A current limitation of the study is that while it is unlikely, it is possible that off-target effects of the gene mutation strategy was responsible for the protection seen in IL-18$^{-/-}$ mice. Therefore, future studies should confirm the role of IL-18 in the development of hypertension with alternate strategies such as anti-IL-18 neutralizing antibodies or by showing that the hypertensive response can be restored in IL-18$^{-/-}$ mice by treatment with recombinant IL-18.

In conclusion, this study has demonstrated for the first time that experimental hypertension is associated with upregulation of several elements of the IL-18 signalling system, particularly on T cells that infiltrate the kidney. Furthermore, this study has demonstrated that IL-18 deficiency is protective against several hallmarks of hypertension such as increased BP, renal inflammation and fibrosis. Overall, these findings reinforce the concept that inflammasome activity is crucial for the development of hypertension and highlights the IL-18 signalling system as a potential target for future anti-hypertensive therapies.

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Figures
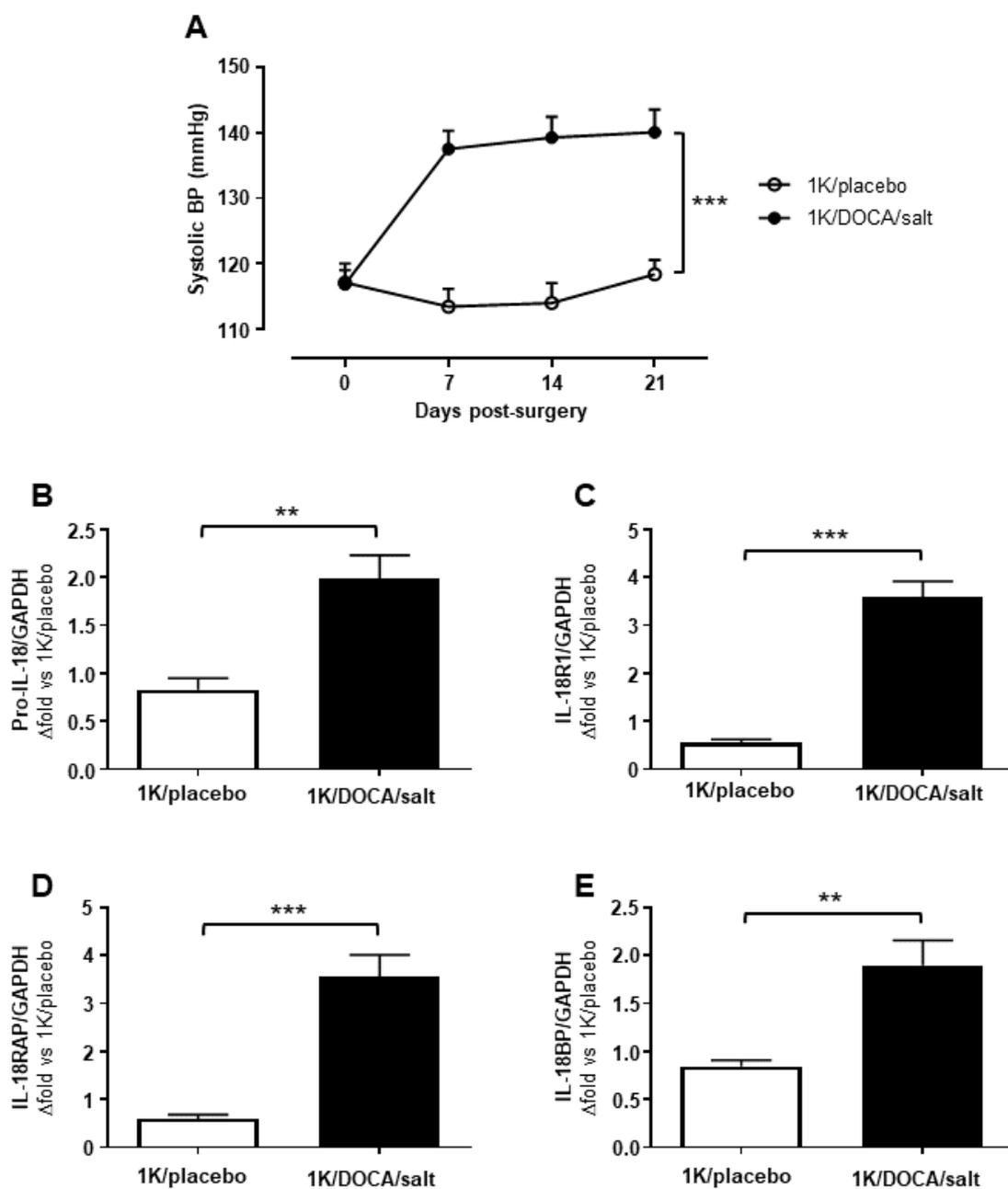
Figure 1

Figure 1: Effect of 1K/DOCA/salt treatment on systolic BP (A) and mRNA expression of IL-18 (B), IL-18R1 (C), IL-18RAP (D) and IL-18BP (E) in the kidneys. Blood pressure was measured via tail-cuff plethysmography for 21 days. Messenger RNA expression in the kidneys was measured with real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM from n<24 per group (for BP data) and n=7-10 per group for all other graphs. **P<0.01 and ***P<0.001 for Bonferroni multiple comparisons test after 2-way ANOVA or Student's unpaired t test.
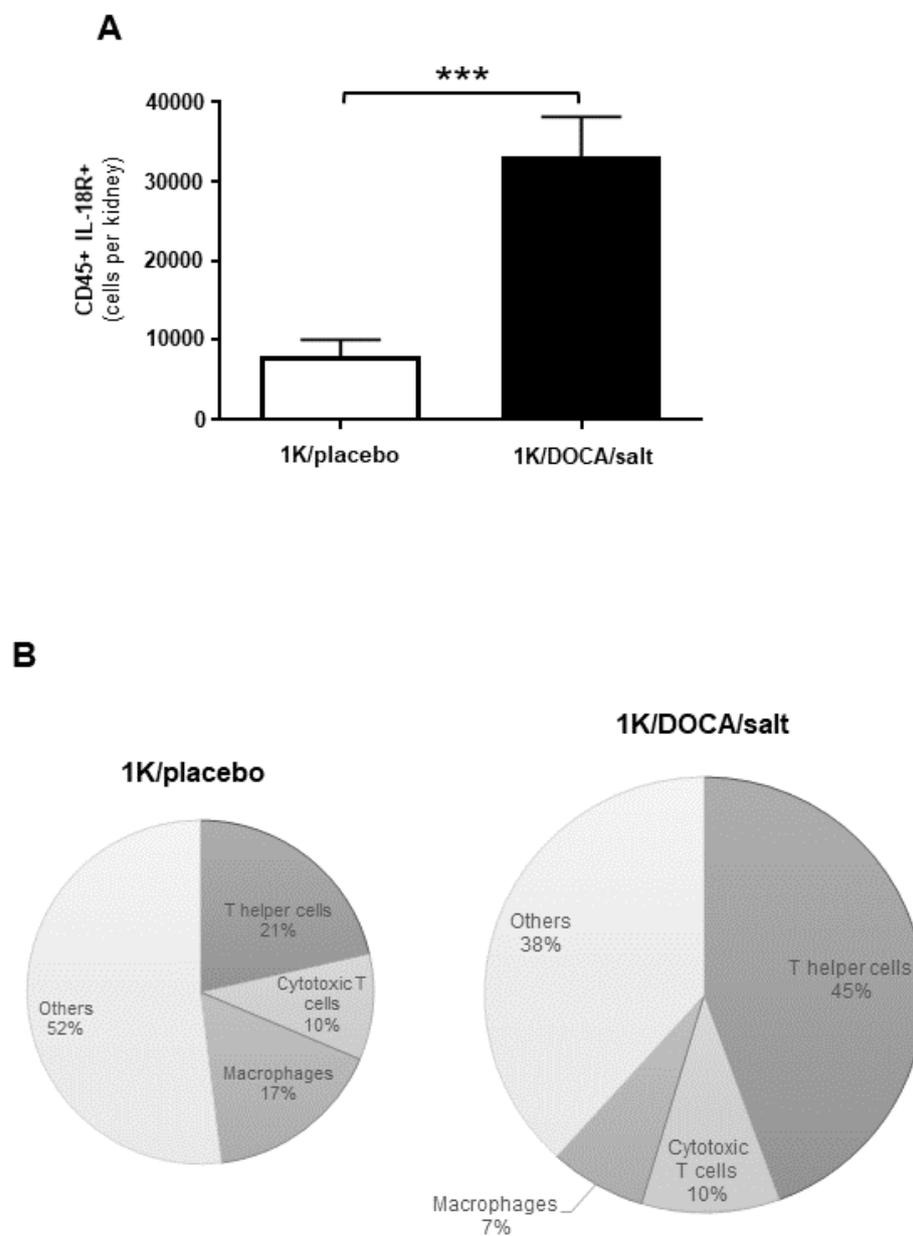
Figure 2

Figure 2: Effect of 1K/DOCA/salt treatment on accumulation of IL-18R-expressing leukocytes in the kidneys. Leukocyte accumulation was quantified using flow cytometric analysis. Panel (A) shows the number of IL-18R1-expressing leukocytes (CD45$^+$IL-18R1$^+$) present in the kidneys of 1K/DOCA/salt-treated versus control mice while panel (B) shows relative proportions of CD4+ T cells, CD8+ T cells and CD11b$^+$F4/80+ macrophages that comprise this IL-18R1$^+$ cell population. Data are expressed as mean ± SEM (n=6-8 per group). ***P<0.001 for Student's t-test. Pie chart is expressed as proportion of total CD45+IL-18R+ expressing population.
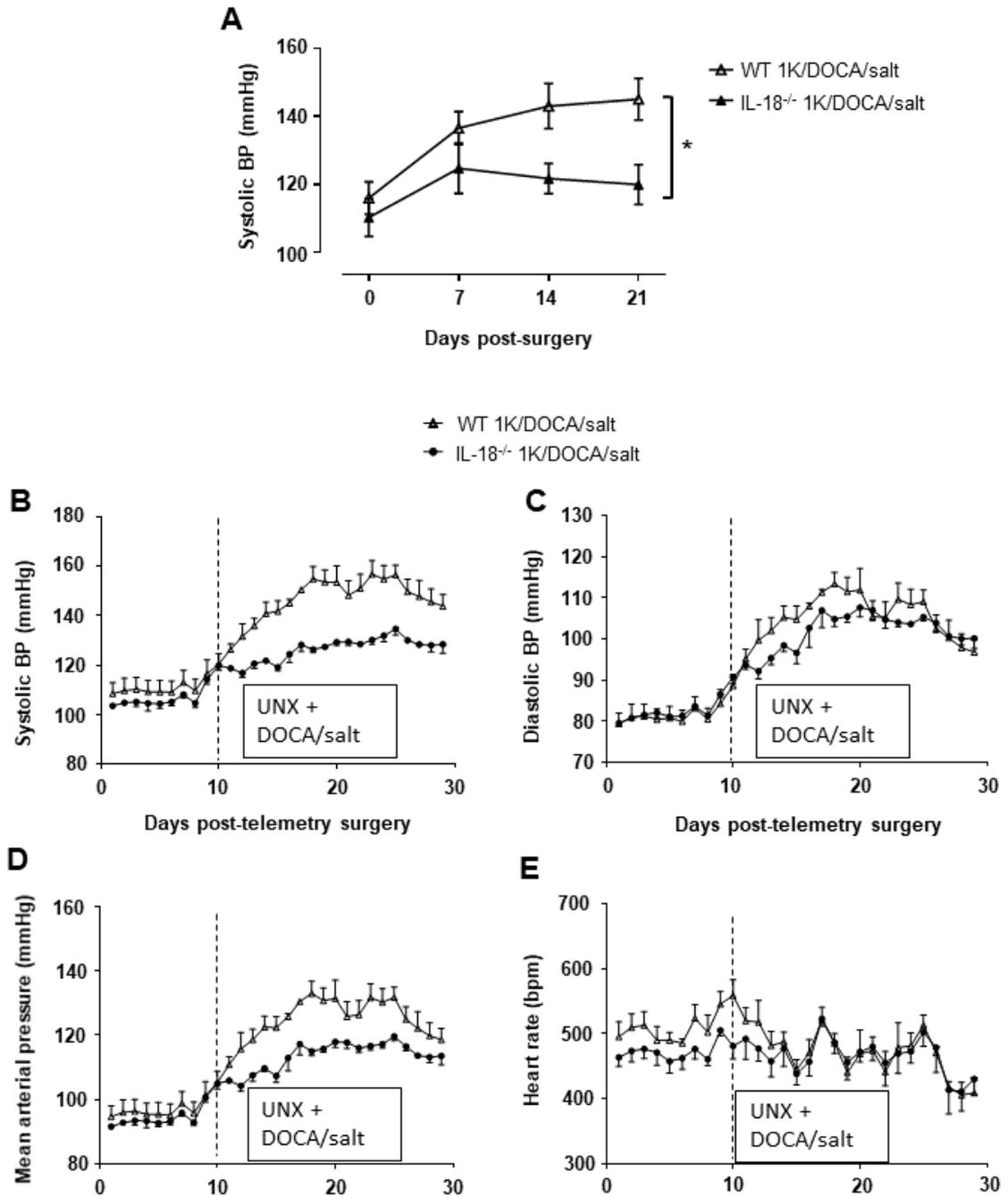
Figure 3

Figure 3: Effect of IL-18 gene-deficiency on 1K/DOCA/salt-induced increases in BP. In panel (A) systolic BP was measured via tail cuff plethysmography, while in panels (B), (C), D) and (E) systolic BP, diastolic BP, mean arterial pressure (D) and heart rate (E), respectively were measured by radiotelemetry. All data are expressed as mean ± SEM (n=3-4 per group). *P<0.05 for 2-way repeated-measures ANOVA.
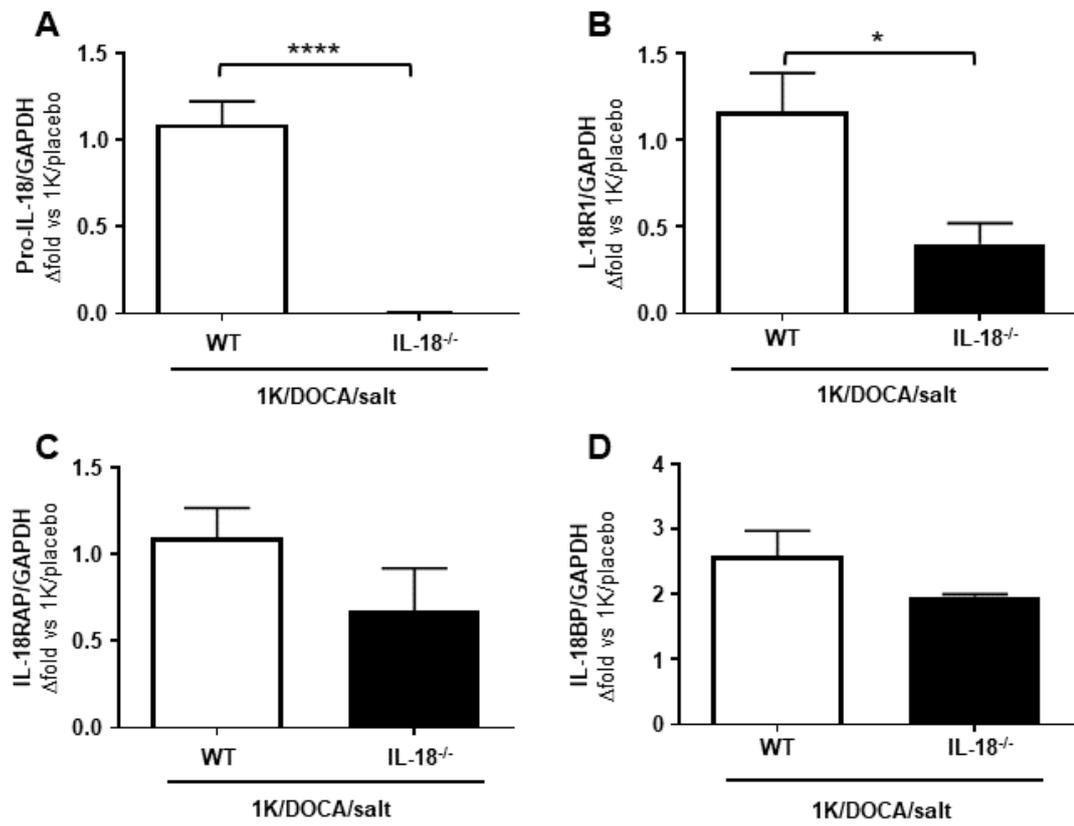
Figure 4

Figure 4: Effect of IL-18 gene-deficiency on renal mRNA expression on elements of the IL-18 signalling system. Messenger RNA expression of IL-18 (A), IL-18R1 (B), IL-18RAP (C) and IL-18BP (D) was measured by real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data expressed as mean ± SEM (n=5 per group) *P<0.05, ****P<0.0001 for Student's unpaired t-test.
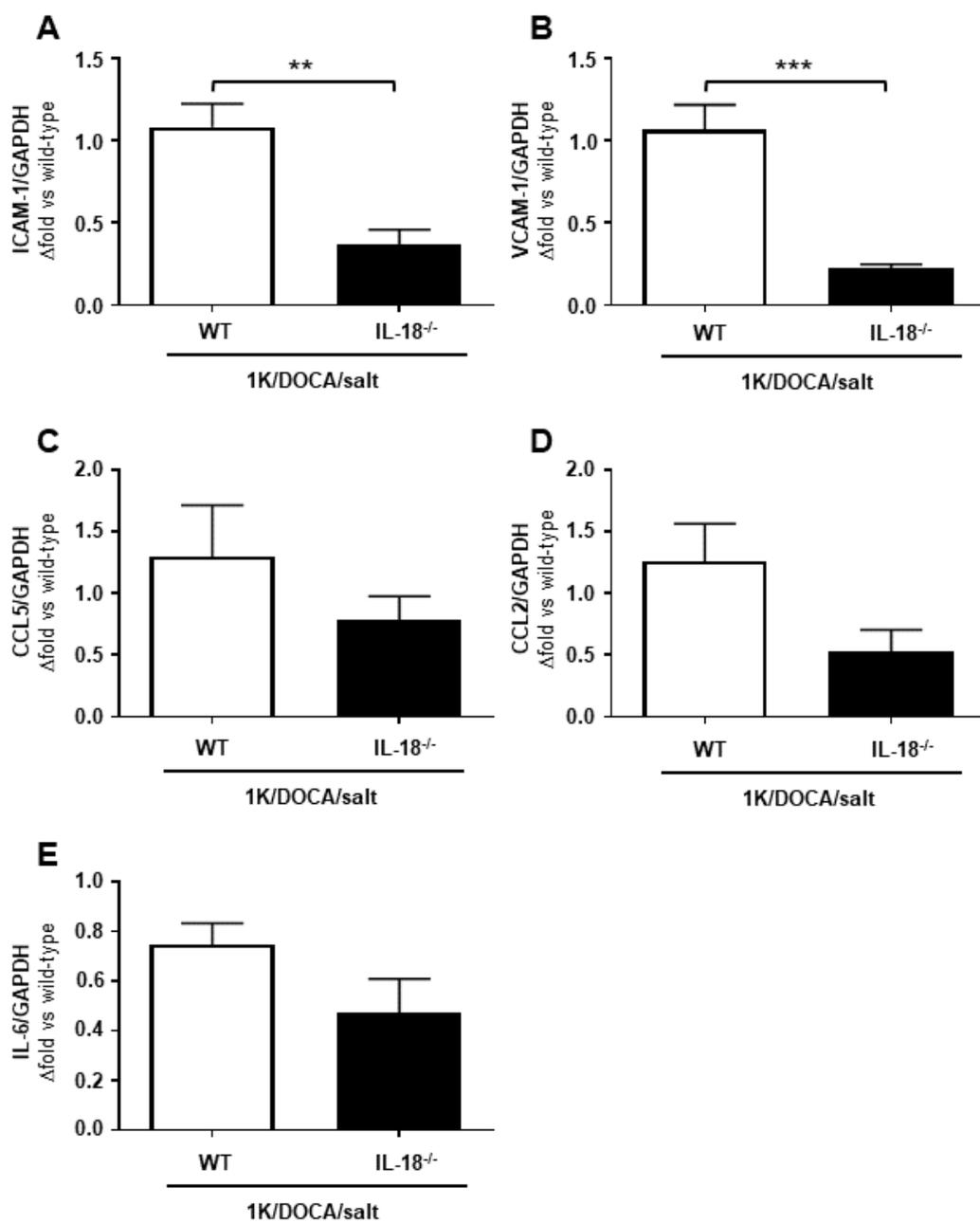
Figure 5

Figure 5: Effect of IL-18 gene deficiency on mRNA expression of inflammatory markers in the kidneys of 1K/DOCA/salt-treated mice. Messenger RNA expression of the adhesion molecules ICAM-1 (A) and VCAM-1 (B); the chemokines CCL5 (C) and CCL2 (D) and the pro-inflammatory cytokine IL-6 was measured by real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data expressed as mean ± SEM (n=5 per group) **P<0.01, ***P<0.001 for Student's unpaired t-test.
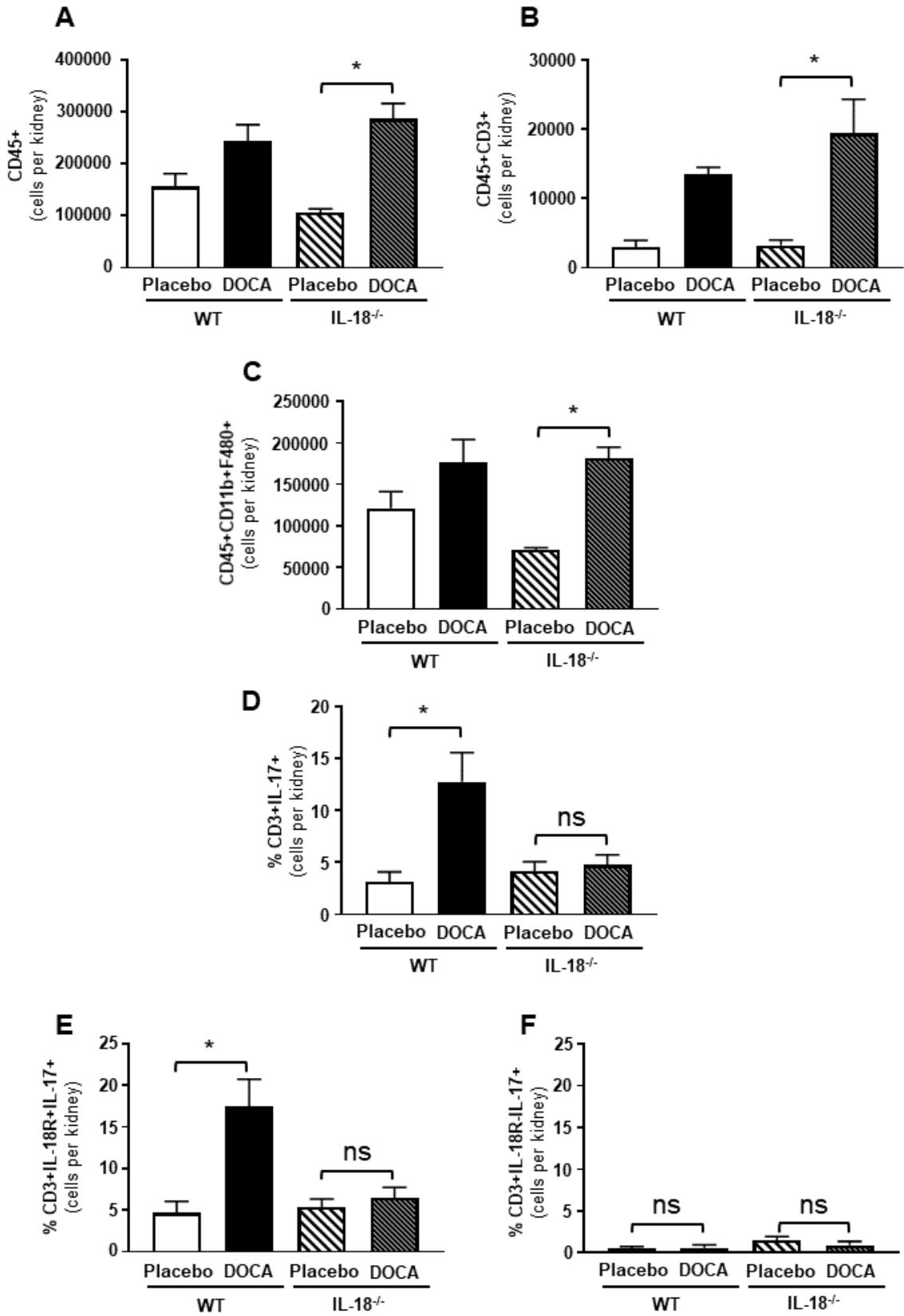
Figure 6

Figure 6: Effect of IL-18 gene deficiency on leukocyte accumulation in the kidneys of 1K/DOCA/salt treated mice. Flow cytometric analysis was used to quantify total leukocytes (CD45+; A), total T cells (CD45$^+$CD3$^+$; B), and macrophages (CD45+CD11b+F4/80+; C) as well as the number of IL-17 producing T cells (CD45+CD3+IL-17+; D) subdivided into those cells expressing IL-18R1 (E) or lacking IL-18R1 (F). Data are expressed as mean ± SEM (n=3-8 per group). *P<0.05 for one-way ANOVA followed by Newman-Keuls multiple comparisons test.
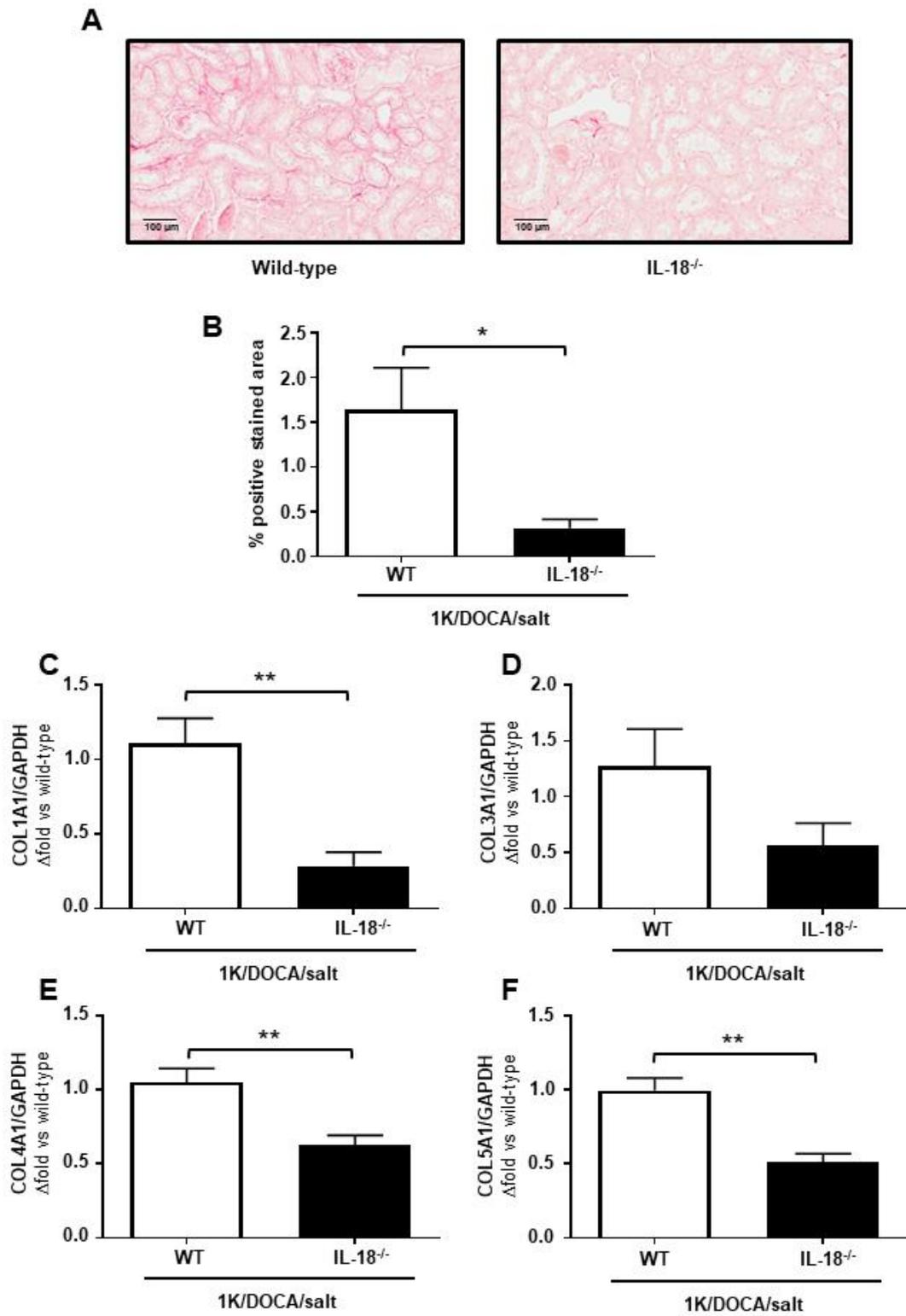
Figure 7

Figure 7: Effect of IL-18 gene deficiency on collagen deposition in the kidneys of 1K/DOCA/salt-treated mice. (A) Representative image (x20 magnification) and (B) quantified group data of Picrosirius red stained kidney sections. Messenger RNA expression of (C) collagen type 1 alpha 1, (D) collagen type 3 alpha 1, (E) collagen type 4 alpha 1, and (F) collagen type 5 alpha 1 was measured by was measured by real-time PCR and quantified using the comparative CT method with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=5 per group). *P<0.05, **P<0.01 for Student's unpaired t-test.
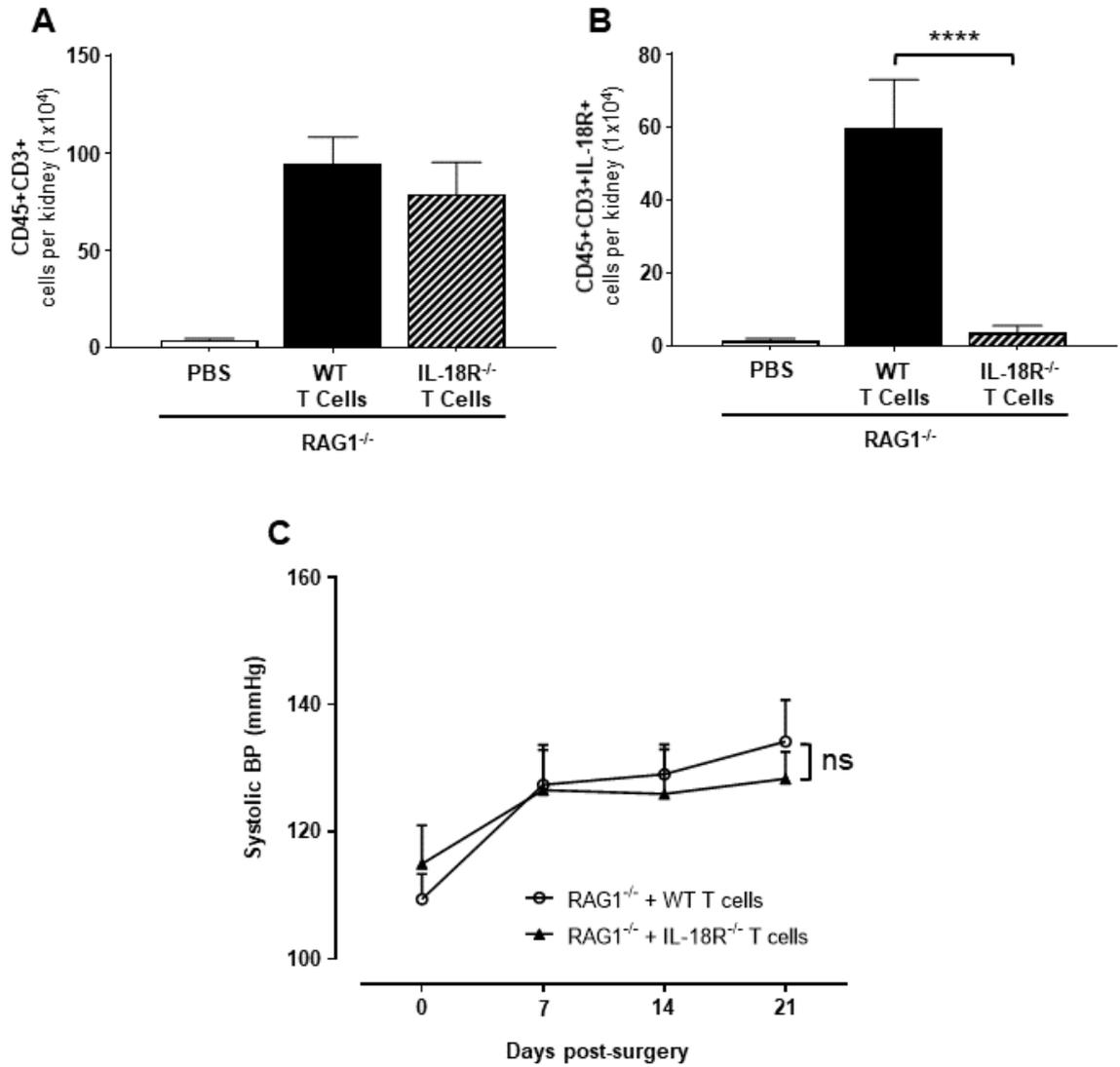
Figure 8

Figure 8: Mice with T cell-specific deficiency of IL-18R are not protected from 1K/DOCA/salt hypertension. (A) Flow cytometric analysis revealed that adoptive transfer of either WT or IL-18R1-deficient T cells in RAG1-/- mice was equally as effective at reconstituting the T cell population in the kidneys. (B) No IL-18R1-expressing T cells were detected in the kidneys of mice that were adoptively transferred with T cells from IL-18R1$^{-/-}$ mice. The degree of hypertension caused by 1K/DOCA/salt treatment was similar between mice with IL-18-deficient versus WT T cells. Data are expressed as mean ± SEM (n=7-10 per group). ****P< for one-way ANOVA followed by Newman-Keuls multiple comparisons test.
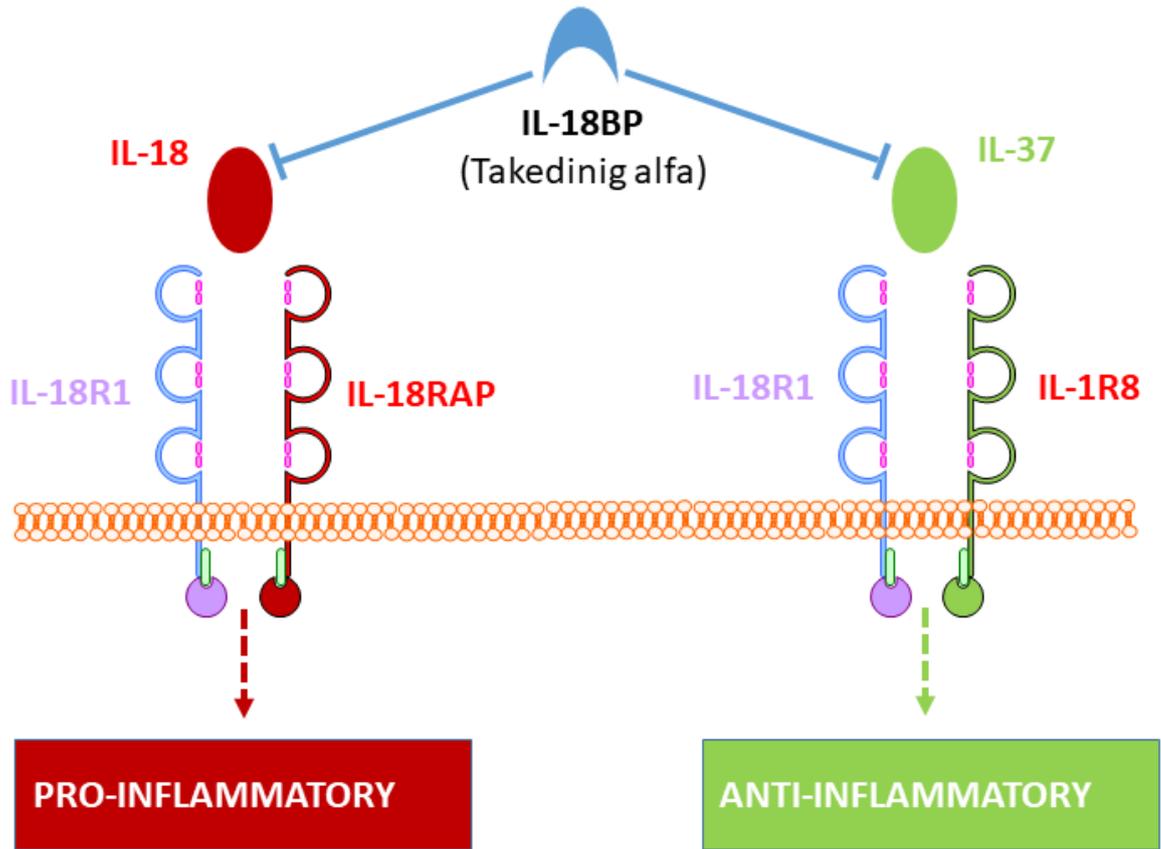
Figure 9

Figure 9: Components of the IL-18 signalling system. Upon binding to the IL-18R1 subunit, IL-18 causes the recruitment of IL-18RAP leading to pro-inflammatory signalling and ultimately hypertension. Conversely, other cytokines that also utilise IL-18R1, such as IL-37, recruit a different suite of adaptor proteins including IL-8R1, which skews the system towards anti-inflammatory signalling. Thus, while targeting of IL-18R1 will likely block both pro- and anti-inflammatory signalling, targeting of IL-18RAP will only block the former. As such, IL-18RAP inhibition is expected to have a greater anti-inflammatory effect that inhibiting IL-18R1.

Chapter 5:

Nitric oxide donors inhibit NLRP3 inflammasome activity in mouse and human macrophages

Abstract

Background: On top of their potent vasodilatory effects, nitric oxide donors are known to have beneficial anti-inflammatory actions. However, the mechanism of these anti-inflammatory actions is still unknown. Inflammasomes are important regulators of pro-inflammatory cytokines such as IL-1β and IL-18 and have emerged as key mediators in many chronic inflammatory diseases. Inflammasome inhibition by nitric oxide donors may explain their anti-inflammatory actions.

Methods: Immortalised mouse bone marrow-derived macrophages (BMDMs) and an immortalised human monocytic cell line, THP-1, were treated with lipopolysaccharide (LPS) (4 h) and adenosine 5'-triphosphate (ATP) (30 min) to prime and activate the NLRP3 inflammasome. Anti-inflammatory mechanisms were determined by co-incubation with the nitric oxide donors SIN-1 or SPER-NO for 4 h. The role of cGMP, NF-κB and other TLRs in this effect were determined by incubation with either ODQ (4 h), lipoteichoic acid (LTA) (4 h), imiquimod (4 h), CPG-ODN (4 h), NBD blocking peptide (4 h) or phorbol myristate acetate (PMA) (4 h). Inflammasome expression was determined by real-time PCR, ELISA and immunohistochemistry.

Results: Nitric oxide donors SIN-1 and SPER-NO were shown to markedly inhibit inflammasome expression both at the mRNA level and protein level as seen with reduced expression of IL-1β in BMDMs and THP-1 cells by ~60% following stimulation with LPS and ATP (n≥5, P<0.05). This effect was comparable when BMDMs were stimulated with other TLR agonists such as imiquimod, CPG-ODN, and LTA. Inhibition of cGMP with ODQ had little effect on reversing nitric oxide mediated inhibition of the inflammasome (n≥5, P<0.05). Furthermore, inhibition of NF-κB signalling with an NBD blocking peptide did little to reverse the effect of nitric oxide on inflammasome activity (n≥5, P<0.05). Lastly, treatment with the NF-κB activator, PMA had little effect on inflammasome expression (n≥5, P<0.05).

Conclusion: This study has shown that nitric oxide donors are potent inhibitors of inflammasome activity and the subsequent production of IL-1β. Although further studies are required to define the precise mechanism of this effect, these findings still provide a potential explanation for the anti-inflammatory properties of nitric oxide donors.

Introduction

Nitric oxide (NO) is a gaseous lipophilic free radical that was initially discovered as an agent that is released by the endothelium to regulate vascular tone in blood vessels [1]. It is now known that in addition to its vasodilatory actions, NO is also an important modulator of inflammation by inhibiting several key leukocyte functions such as neutrophil and monocyte adhesion, immune cell chemotaxis and superoxide generation [2,3]. However, despite knowledge of these many and diverse effects of NO on key inflammatory processes, there is little information on the molecular mechanisms involved.

Inflammasomes have emerged as key mediators of inflammation in many chronic diseases such as rheumatoid arthritis, type II diabetes, atherosclerosis, gout, and Crohn's disease [4–6]. Inflammasomes are multimeric protein complexes expressed in immune and non-immune cells that regulate the production of pro-inflammatory cytokines such as IL-1β, and IL-18 in response to pathogen associated molecular patterns (PAMPS) or host-derived danger-associated molecular patterns (DAMPS) [4,7–9]. PAMPS are small distinguishing molecular 'signatures' expressed by a wide range of pathogens and include molecules such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [10,11]. DAMPS are signals that are intrinsic to the host and indicate disruption to normal cell function. These include molecules such as hyaluronan, amyloid β, and high levels of extracellular ATP [12,13].

The NLRP3 inflammasome complex consisting of a NOD-like receptor protein, NLRP3; an adaptor protein, ASC; and pro-caspase-1, is the most well characterised member of the inflammasome family. Activation of the NLRP3 inflammasome is a two-step process involving (1) transcriptional upregulation of various subunits of the inflammasome complex as well as the "pro" forms of IL-1β, and IL-18 (this process is often referred to as "priming") and (2) NOD-like receptor (NLR) dependent "activation" of inflammasome assembly leading to the clustering and subsequent auto-cleavage of pro-caspase-1 into active caspase-1,

which is then able to cleave pro-IL-1β, and pro-IL-18 into their active forms, IL-1β and IL-18 [4,8].

Previous studies have shown that NO may partially mediate its anti-inflammatory effects by inhibition of the transcription factor, NF-κB [14–17]. NF-κB is an important inflammatory transcription factor and plays a crucial role in regulating levels of various pro-inflammatory cytokines and proteins such as IL-6 [18], and the inducible forms of NO synthase (iNOS) [19]. Indeed, as a transcriptional activator of NLRP3, caspase-1, pro-IL-1β, and pro-IL-18, NF-κB is also implicated in the initial "priming" phase of NLRP3 inflammasome activation [4,7]. However, while a study conducted by Mao *et al.* (2013) showed that NO donors can inhibit oligomerisation of the NLRP3 complex, no studies have directly shown whether the inhibitory effects of NO on NF-κB actually translate to a reduction in NLRP3 priming. Therefore, in the present study, we sought to determine whether NO has any direct inhibitory effect on priming and/or activation of the NLRP3 inflammasome in macrophages, and if so by what mechanism it achieves such effects.

Methods

Cell culture

Immortalised bone marrow-derived macrophages (BMDMs), originally harvested from C57BL/6J mice, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). An immortalised mouse bone marrow-derived macrophage cell line that had been stably transduced with mCerulean tagged ASC was also cultured in DMEM with 10% FBS. The immortalised human monocytic cell line, THP-1, was cultured in high glucose RPMI 1640 medium supplemented with 10% FBS. Wild-type BMDMs and mCerulean tagged BMDMs were passaged every three days at a 1:10 ratio in DMEM with 10% FBS. THP-1 cells were passaged every three days to contain 1×10^5 cells in each flask. For the extraction of RNA, both wild-type BMDMs and THP-1 cells were seeded at a density of 5×10^5 cells per well in 6-well plates. THP-1 cells were first treated with 100 nM of phorbol 12,13-dibutyrate (PDBu) (Sigma, USA) for 24 h to allow differentiation into macrophages. Wild-type BMDMs were allowed at least 12 h to adhere to the plate surface. For the visualisation of ASC specks in BMDMs expressing mCerulean tagged ASC, cells were seeded at a density of 2.5×10^4 cells/100 µl per well in Nunc™ Lab-Tek™ 8 well chamber slides for 24 h in DMEM + 10% FBS.

Cell culture treatments

Initial priming of the inflammasome was achieved in all cell lines by incubation in 1 µg/ml LPS (Sigma) for 4 h. To serve as controls, some cells were left un-primed for the duration of the experiment. Subsequent assembly and activation of the inflammasome was achieved by further incubation in 3 mM adenosine 5′-triphosphate (ATP) (Sigma) for the final 30 min of the incubation period. To assess the effects of NO donors on inflammasome priming/activation, SPER-NO (Cayman Chemical, USA) and SIN-1 (Cayman Chemical, USA) were added to cells at the same time as LPS at concentrations ranging from 10 - 500 µM. Where applicable, the cyclic GMP inhibitor, ODQ (Cayman Chemical, USA) or the NO

scavengers, hydroxocobalamin hydrochloride (HC) (Sigma, USA) and carboxy-PTIO (PTIO) (Cayman Chemical, USA) were also added to cells at the same time as LPS and SPER-NO, at concentrations of 10 µM, 200 µM and 100 µM respectively. TLR2, TLR7 and TLR9 activation was achieved by incubating cells for 4 h with lipoteichoic acid (LTA; 1 µg/ml) (Invivogen, USA), imiquimod (20 µg/ml) (Invivogen, USA), and CpG Oligodeoxynucleotide (CpG ODN) (2 µM) (Invivogen, USA), respectively. As with the LPS studies, SPER-NO was added to cells at the same time as these TLR agonists. To determine if NO mediated its effects on inflammasome priming by inhibition of NF-κB, an NBD (NEMO-binding domain) blocking peptide (Enzo Life Sciences, USA) was incubated for 4 h at 25 µM in conjunction with LPS and SPER-NO treatment as previously described. Furthermore, activation of NF-κB was achieved by using phorbol 12-myristate 13-acetate (PMA) at concentrations of 1 nM, 10 nM, and 100 nM for 4 h to determine the effect of NF-κB activity on pro-IL-1β expression.

Measurement of mRNA expression levels

RNA was extracted from cells 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) or Qiaxpert System (Qiagen, USA). RNA was reverse transcribed to cDNA for use as a template in real-time PCR using a High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA). Real-time PCR was conducted using Taqman® primers and probes for NLRP3 and pro-IL-1β and the house keeping gene GAPDH (Taqman Gene Expression Assays, Applied Biosystems, USA). Real-time PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Australia). Fold-change changes in mRNA expression relative to control samples were determined using the comparative CT method [20].

Chapter 5: Nitric oxide donors inhibit NLRP3 inflammasome in macrophages

Immunohistochemistry for ASC specking

Twenty four hours after seeding of mCerulean tagged ASC BMDMs in Nunc™ Lab-Tek™ 8 well chamber slides, cells were treated with LPS, ATP and SPER-NO as outlined above. After the treatment period, cells were washed with phosphate-buffered saline (PBS) (0.01 M) and fixed in 4% paraformaldehyde (PFA) for 15 min. Cells were then washed three times for 15 min with PBS. After physical removal of the plastic walls of the chamber slide system, cover slips were mounted onto the microscope slides with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, USA). Slides were viewed and photographed on a Nikon Upright inverted confocal fluorescence microscope (Nikon D-eclipse C1) at 20x magnification.

Enzyme-linked Immunosorbent Assay for IL-1β levels

Cell media was collected after the various treatment periods and placed in microcentrifuge tubes, snap frozen in liquid N_2 and stored at -80°C. Prior to conducting the assays, the media was centrifuged at 1500 RPM for 5 min to pellet and remove any debris or dead cells. Colorimetric ELISA kits for mouse IL-1β were purchased from Elisakit.com (Australia). The assays were performed according to manufacturer's instruction in 96-well microtiter plates. Briefly, a concentration range of serially diluted standards (supplied by the manufacturer) and cell medium samples were loaded directly into the wells of the microtiter plates in duplicate and incubated at room temperature for 2 h. Wells were washed with washing buffer prior to the addition of a secondary detection antibody consisting of anti-mouse IL-1β conjugated to biotin and incubated for 2 h. Wells were then washed and streptavidin-HRP was added and incubated for 20 min at room temperature. All wells were then washed and a substrate containing hydrogen peroxide and tetramethyl benzidine was added and incubated for 15 min or prior to overdevelopment of the plate. Reactions were stopped using 0.5 M of H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader (Versa max Tunable Microplate Reader; Biostrategy, Australia). The amount of IL-1β in

samples was estimated by comparing the optical density (OD) values of the samples with the OD values of the standards.

Statistical analysis

Data are expressed as mean ± SEM. Data were analysed by one-way ANOVA followed by Newman-Keuls *post hoc test* where appropriate. $P < 0.05$ was considered to be statistically significant. Data were graphed and analysed using Graphpad Prism Software v6.04

Results

SIN-1 and SPER-NO inhibits LPS stimulated increases in NLRP3 and pro-IL-1β expression in immortalised mouse BMDMs and THP-1 cells

Consistent with previous reports [8,21,22], LPS treatment caused an increase in pro-IL-1β mRNA expression in both mouse and human macrophage cell lines (Fig 1B & 1D). LPS treatment also increased NLRP3 mRNA expression by ~13 fold in immortalised BMDMs but not in THP-1 cells (Fig 1A & 1C). The NO generator, SIN-1, had no effect on basal expression of NLRP3 or pro-IL-1β in unstimulated cells (Fig 1). However, SIN-1 blunted the LPS-induced increases in pro-IL-1β expression in both THP-1 cells and immortalised BMDMs. There was also a trend for SIN-1 to reduce NLRP3 expression in LPS-treated BMDMs and THP-1 cells, although these effects did not reach statistical significance (Fig 1A, 1B & 1D). A similar profile of inhibitory effects of SIN-1 on NLRP3 and pro-IL-1β expression, was observed in cells stimulated with the combination of the priming agent (LPS) and the inflammasome activator, ATP.

A chemically distinct NO donor, SPER-NO was found to be even more effective than SIN-1 at inhibiting inflammasome priming. Indeed, SPER-NO inhibited LPS-stimulated induction of pro-IL-1β by ~90% in both mouse and human macrophage cell lines. It also blocked LPS-induced upregulation of NLRP3 by ~50% in both the mouse and human cell lines, although this effect only reached statistical significance in the former (Fig 1).

SPER-NO concentration dependently reduces mRNA expression of NLRP3 and pro-IL-1β in immortalised mouse BMDMs.

To determine the concentration range over which NO donors inhibited inflammasome priming, we examined the effects of varying concentrations of SPER-NO (10 - 250 µM) on LPS-induced increases in NLRP3 and pro-IL-1β expression in mouse BMDMs. While the lower concentrations of 10 µM and 25 µM had no inhibitory effects on LPS-induced

increases in NLRP3 and pro-IL-1β expression, both the 100 µM and 250 µM treatments were highly effective, reducing LPS-induced NLRP3 expression by ~40% and ~50% respectively; and pro-IL-1β expression by ~50% and ~90%, respectively (Fig 2A & 2B).

SIN-1 and SPER-NO treatment prevents ATP-dependent assembly and activation of the NLRP3 inflammasome

Preliminary immunofluorescence studies revealed that there were no ASC-specks present in unstimulated mCerulean-tagged ASC BMDMs (Fig 3A). There was also no evidence of ASC specks in mCerulean-tagged ASC BMDMs stimulated with LPS alone, or with a combination of LPS plus SPER-NO (Fig 3A). However, ASC specks *were* clearly visible in cells exposed to the combination of LPS plus ATP, suggesting that this treatment indeed stimulated inflammasome assembly (Fig 3A). Further treatment of the LPS plus ATP stimulated mCerulean-tagged ASC BMDMs with SPER-NO greatly reduced the number of cells with ASC specks (Fig 3A), consistent with the concept that the NO donor inhibited inflammasome assembly. Also consistent with an inhibitory effect on inflammasome assembly/activation were our findings from ELISAs measuring IL-1β in the growth medium of THP-1 cells and mouse BMDMs after treatment with various combinations of NO donors, LPS and ATP. In THP-1 cells, LPS alone caused a 100-fold increase in IL-1β levels in the media and this was inhibited by ~50% by SIN-1, although this effect did not reach statistical significance (Fig 3B). The combination of LPS plus ATP caused an even greater increase in IL-1β generation (~250-fold) and again this effect was inhibited by ~50% by SIN-1 (Fig 3B). In mouse BMDMs, LPS alone had only a minimal effect on IL-1β production; however, the combination of LPS plus ATP stimulated a ~40-fold increase in levels of IL-1β in the media (Fig 3C). This effect was almost abolished by co-treatment with SPER-NO (Fig 3C).

<u>NO does not mediate its effects on the inflammasome through activation of soluble guanylyl cyclase (sGC).</u>

In a separate series of experiments, we investigated whether the inhibitory effects of NO donors on inflammasome priming and activation could be inhibited by an sGC inhibitor, ODQ. As per our earlier findings, LPS (alone or in combination with ATP) caused a marked increase in mRNA expression levels of pro-IL-1β in mouse BMDMs and these effects were blocked ~90% by SPER-NO (Fig 4A & 4B). Pre-treatment of cells with ODQ, at a concentration that was 10-fold higher than its IC_{50} against sGC, failed to reverse the inhibitory effects of SPER-NO on LPS-induced increases in pro-IL-1β mRNA expression (Fig 4A & 4B). An NO scavenger compound, carboxy-PTIO, appeared to partially reverse the inhibitory effects of SPER-NO on LPS-induced upregulation of pro-IL-1β, but this effect did not reach statistical significance (Fig 4A & B).

A similar profile of responses was observed when we measured IL-1β production by LPS plus ATP stimulated cells. The combination of LPS plus ATP caused a 40-fold increase in IL-1β production, which was markedly inhibited by SPER-NO (Fig 4C). Again, ODQ failed to reverse this inhibitory effect of SPER-NO (Fig 4C). As per our findings with mRNA expression, carboxy-PTIO appeared to partially reverse the inhibitory effects of SPER-NO on LPS plus ATP-induced IL-1β production, but again this effect failed to reach statistical significance (Fig 4C).

To investigate whether a mechanistically distinct NO scavenger might be more effective at reversing SPER-NO mediated inhibition of LPS-induced increases in inflammasome priming, we examined the effects of hydroxocobalamin. For these studies, we lowered the concentration of SPER-NO to 100 µM such that the stoichiometric ratio of NO produced: NO scavenged would be more favourable (i.e. 2:1). SPER-NO, at a concentration of 100 µM, reduced the LPS-induced increase in pro-IL-1β by 30-50%. Again, while there was a

trend for the NO scavenger to reverse the inhibitory effects of SPER-NO by ~30%, this did not reach significance (Fig 4D).

NO donors inhibit inflammasome priming irrespective of the stimulus

BMDMs stimulated with other TLR agonists such as LTA for TLR2, imiquimod for TLR7, and CpG ODN for TLR9, displayed marked increases in pro-IL-1β expression, indicating that inflammasome priming occurs downstream of all of these TLR subtypes (Fig 5B-D). However, it should be noted that all of these TLR agonists were an order of magnitude weaker that LPS in their ability to stimulate pro-IL-1β expression (Fig 5A-D). Regardless, the NO donor, SPER-NO, was effective at inhibiting inflammasome priming by all of these TLR agonists (Fig 5A-D).

Activation of NF-κB and NO does not affect the mRNA expression of pro-IL-1β in immortalised mouse BMDMs

As mentioned previously, the transcription factor, NF-κB is a master regulator of inflammation and has been suggested to play an important role in inflammasome priming through transactivation of various inflammasome subunits and pro-IL-1β. Moreover, it has been suggested that the anti-inflammatory properties of NO may be mediated in part through the direct inhibitory effect on NF-κB. Therefore, to determine if inhibition of NF-κB might underlie the inhibitory actions of NO on inflammasome priming, we first attempted to confirm that this transcription factor indeed plays a role in LPS-induced upregulation of pro-IL-1β in our model system. Surprisingly, an inhibitor of NF-κB - NBD blocking peptide - had no effect on the LPS-stimulated increase in expression of pro-IL-1β in BMDMs (Fig 6A). In the same experiment, SPER-NO caused a 60% reduction in LPS-stimulated upregulation of pro-IL-1β, and this inhibitory effect was not affected by co-treatment with the NBD blocking peptide (Fig 6A).

The above findings argue against a role for NF-κB in LPS-induced inflammasome priming in mouse BMDMs. Consistent with this, PMA, which is known to be a powerful activator of

NF-κB via its ability to stimulate protein kinase C activity, was ineffective at inducing expression of pro-IL-1β in BMDMs (Fig 6B). Hence, collectively, these findings suggest that NF-κB is not an important mediator of inflammasome priming in BMDMs, and hence that NO donors must be targeting other signalling elements in the 2nd messenger pathway that leads to upregulation of pro-IL-1β.

Discussion

The key finding from this study is that NO donors inhibit priming of the NLRP3 inflammasome in both mouse and human macrophages. This effect appears to be independent of sGC and is applicable to inflammasome priming in response to at least four different TLR pathways. A surprising finding from this study was that the transcriptional upregulation of IL-1β by TLR activation (and thus the inhibitory effects of NO donors) seems to occur independently of NF-κB activity.

It has been shown previously that NO can modulate the activity of NF-κB and thereby affect expression levels of several pro-inflammatory cytokines [14–17]. Given that NF-κB has been implicated in inflammasome priming, an obvious next step was to examine whether NO might also inhibit this process. Indeed, in the present study, we showed that two chemically distinct NO donors had direct inhibitory effects on the transcriptional upregulation of both NLRP3 and pro-IL-1β, and that these effects were irrespective of the TLR subtype that was stimulated to evoke inflammasome priming. The fact that NO donors were effective at inhibiting inflammasome priming irrespective of TLR subtype suggests that the target of NO is likely to be a signalling element that is common to all of these receptors, with NF-κB being an obvious candidate. Thus, it came as a surprise to observe that an NBD blocking peptide – which blocks NF-κB mediated transcription by inhibiting the association of the NF-κB essential modulator (NEMO) to the IκB kinase complex thus directly inhibiting the translocation of NF-κB to the nucleus [23] – was ineffective at preventing LPS-induced inflammasome priming. This was despite the fact that the concentration of the NBD blocking peptide used was 5-times higher than its reported IC_{50} for IκK [24]. Consistent with this, a PKC activator, PMA, which is well established to stimulate NF-κB in other cell types [25,26], failed to induce inflammasome priming. The implications of these findings are two-fold: (1) that TLR-mediated inflammasome priming in mouse BMDMs occurs via a non-classical mechanism that is independent of NF-κB; and (2) an inhibitory effect on NF-κB is not an explanation for how NO donors suppress inflammasome priming. A potential caveat to

these conclusions is that the NF-κB inhibitor/activator tools used in this study failed to modulate the activity of the transcription factor. We did attempt to measure NF-κB activity directly by using a commercially available NF-κB reporter assay but, due to technical difficulties, we were unable to generate any meaningful data. Thus, while we acknowledge that this as a limitation of the present study and recognise the importance of confirming inhibition/activation of NF-κB in our cell system, it is interesting to speculate on other potential mechanisms by which NO may be reducing inflammasome priming. While the current study has focused on NF-κB mediated upregulation of IL-1β transcription, other transcription factors such as Ccaat-enhancer-binding proteins (C/EBP) [21] and Activator Protein 1 (AP-1) [12,27] have been shown to upregulate the expression of IL-1β and might have therefore been the targets of the inhibitory effects of NO donors seen here.

Irrespective of the precise target of the NO donors, we can draw some conclusions about the molecular mechanisms that were likely to underlie their inhibitory effects on inflammasome priming. NO primarily mediates its effects through one of two mechanisms. The first involves activation of sGC, an enzyme that regulates the production of cyclic guanosine 3', 5'-monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) [28]. cGMP is an important second messenger molecule that is known to modulate the activity of several transcription factors thus leading to changes in the expression levels of many genes [29,30]. The second mechanism by which NO may mediate its effects in cells is via the nitrosylation of cysteine residues on proteins. This reversible process leads to changes in the tertiary structure of proteins which may alter their function [31]. To determine which of these two pathways was likely to be responsible for inhibition of LPS-induced inflammasome priming, we employed an inhibitor of sGC activation, ODQ, at a concentration far exceeding its reported IC_{50} against the enzyme [32]. ODQ did not affect the ability of the NO donors to inhibit LPS-induced inflammasome priming in mouse BMDMs, suggesting that the sGC/cGMP pathway is not involved. This would imply that the inhibitory effects of the NO donors are more likely to be due to S-nitrosylation. To explore

this concept further, it would be interesting to investigate if strategies aimed at blocking S-nitrosylation in BMDMs obviate the inhibitory effects of NO donors against inflammasome priming. Such strategies might first involve using comprehensive proteomic approaches aimed at identifying the signalling molecules downstream of TLR activation that are S-nitrosylated. A common approach for investigating this is the biotin switch approach, which involves the reduction of S-nitrosylated cysteine residues with ascorbate followed by a reaction with biotin-(N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide) [33,34]. Avidin-based capture of the target proteins can then be employed to determine either via antibody-based or mass spectrometry which proteins are S-nitrosylated [33,34]. Having identified target cysteine residues on proteins of interest, it would then be possible to mutate such residues (e.g. serine/alanine replacement) to determine if this blocks the inhibitory actions of NO donors on inflammasome priming [33,34].

In this study, we also employed two NO scavengers, carboxy-PTIO and hydroxocobalamin, to confirm that the effects of SPER-NO on inflammasome priming was indeed due to NO release. Although each compound appeared to have some effect on SPER-NO-mediated inhibition of inflammasome priming, the degree of reversal was only modest. This is perhaps not surprising when one considers (a) the concentration of inhibitors used; (b) the stoichiometry of NO release by SPER-NO; and (c) the stoichiometry of the subsequent reaction between NO and each inhibitor. In brief, each molecule of SPER-NO releases two molecules of NO [35], while each molecule of hydroxocobalamin and carboxy-PTIO, scavenges one molecule of NO. Thus, when equimolar concentrations of SPER-NO and each inhibitor are used (as per the present study), the concentration of NO in solution would only be reduced by half (at most).

Another observation in the present study was that in addition to inhibiting transcription of the inflammasome components, NO donors also appeared to inhibit inflammasome assembly (i.e. ASC speck formation [36]) and the subsequent release of mature/active IL-1β. A likely explanation for these observations was that NO-donor mediated inhibition of

NLRP3 and pro-IL-1β transcription left the cells devoid of enough of the protein components required to form a functional inflammasome complex. However, we cannot rule out the possibility that NO donors also caused post-translational modifications on one or more of the inflammasome subunits, thereby directly inhibiting assembly/activation. Thus, in future studies it will be interesting to determine if one or more of the inflammasome subunits themselves are susceptible to post-translational modification (e.g. S-nitrosylation) following exposure to NO donors.

In light of the above findings, it is interesting to note that NO donors are already used to treat cardiovascular diseases. Owing to their powerful vasodilator effects on the coronary vasculature and their ability to reduce cardiac pre-load, NO donors are indicated for the treatment of angina [37–39]. The present findings suggest that NO donors could also be of benefit for the treatment of hypertension. Not only would they be expected to directly lower BP by reducing cardiac output (venodilation) and peripheral resistance (arteriolar dilation) [40] but, through inhibition of inflammasome signalling, NO donors may also target the underlying inflammatory processes which lead to damage of key BP-regulating organs such as blood vessels, kidneys, heart, and brain.

In conclusion, this study has shown for the first time that NO donors are powerful inhibitors of inflammasome priming and the subsequent production of pro-inflammatory IL-1β by both mouse and human macrophages. Although further studies are required to define the precise mechanism of action of NO-mediated inhibition of inflammasome priming/activity, our findings provide a potential explanation for the long established anti-inflammatory properties of NO. This study also highlights the potential for exploiting the dual inhibitory effects of NO donors on BP and inflammasome activity as future therapies for hypertension and its sequelae of vascular and renal inflammation.

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Figures
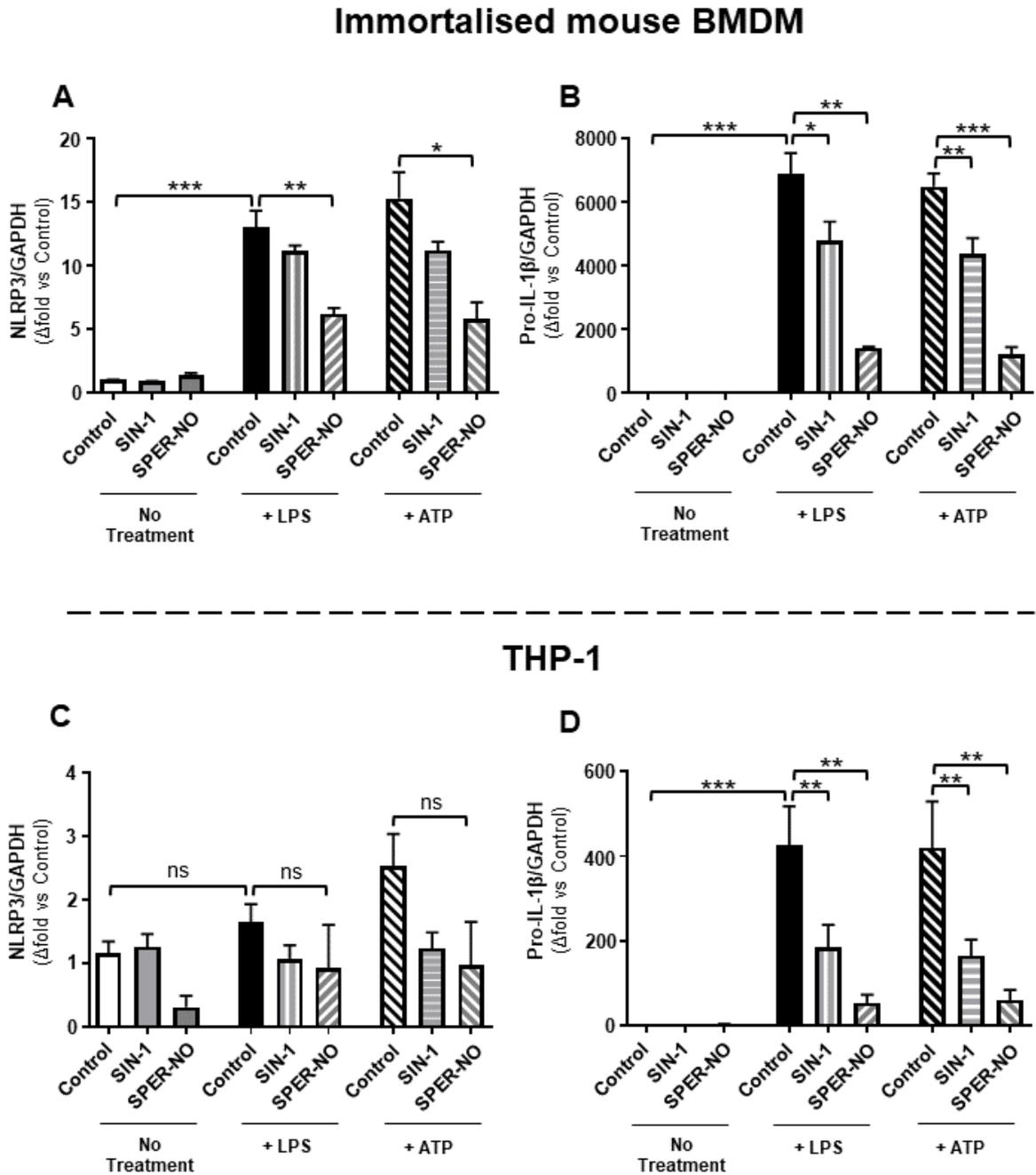
Figure 1

Figure 1: Effect of SIN-1 and SPER-NO on LPS-induced priming of the NLRP3 inflammasome in immortalised mouse BMDMs and THP-1 cells. BMDMs and THP-1 cells were stimulated with LPS (1 µg/ml) and/or ATP (3 mM) and co-treated with either SIN-1 (500 µM) or SPER-NO (250 µM). mRNA expression of NLRP3 and pro-IL-1β in immortalised mouse BMDMs (A and B) and human THP-1 cells (C and D) was measured with real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=3-9 per group). *P<0.05 **P<0.01 ***P<0.001 for one-way ANOVA followed by Newman Keuls multiple comparisons test.
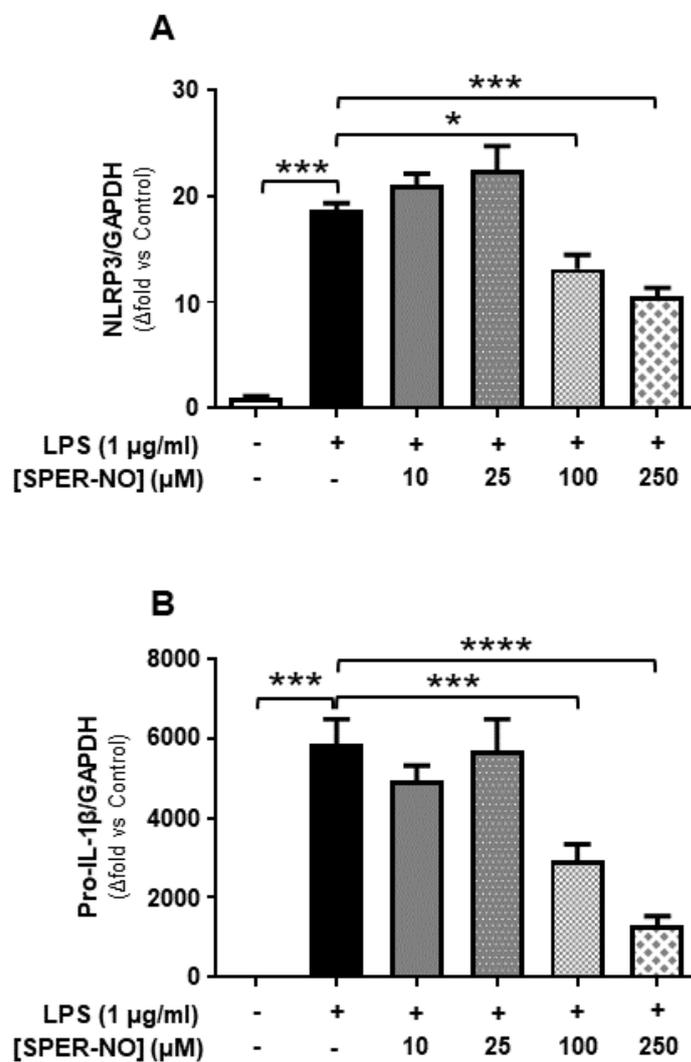
Figure 2

Figure 2: Effect of different concentrations of SPER-NO on LPS-induced upregulation of NLRP3 and pro-IL-1β in mouse BMDMs. BMDMs were stimulated with LPS (1 µg/ml) and treated with a range of SPER-NO concentrations (10 - 250 µM). mRNA expression of NLRP3 (A) and pro-IL-1β (B) was measured with real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=7 per group). *P<0.05, ***P<0.001, ****P<0.0001 for one-way ANOVA followed by Newman Keuls multiple comparisons test.
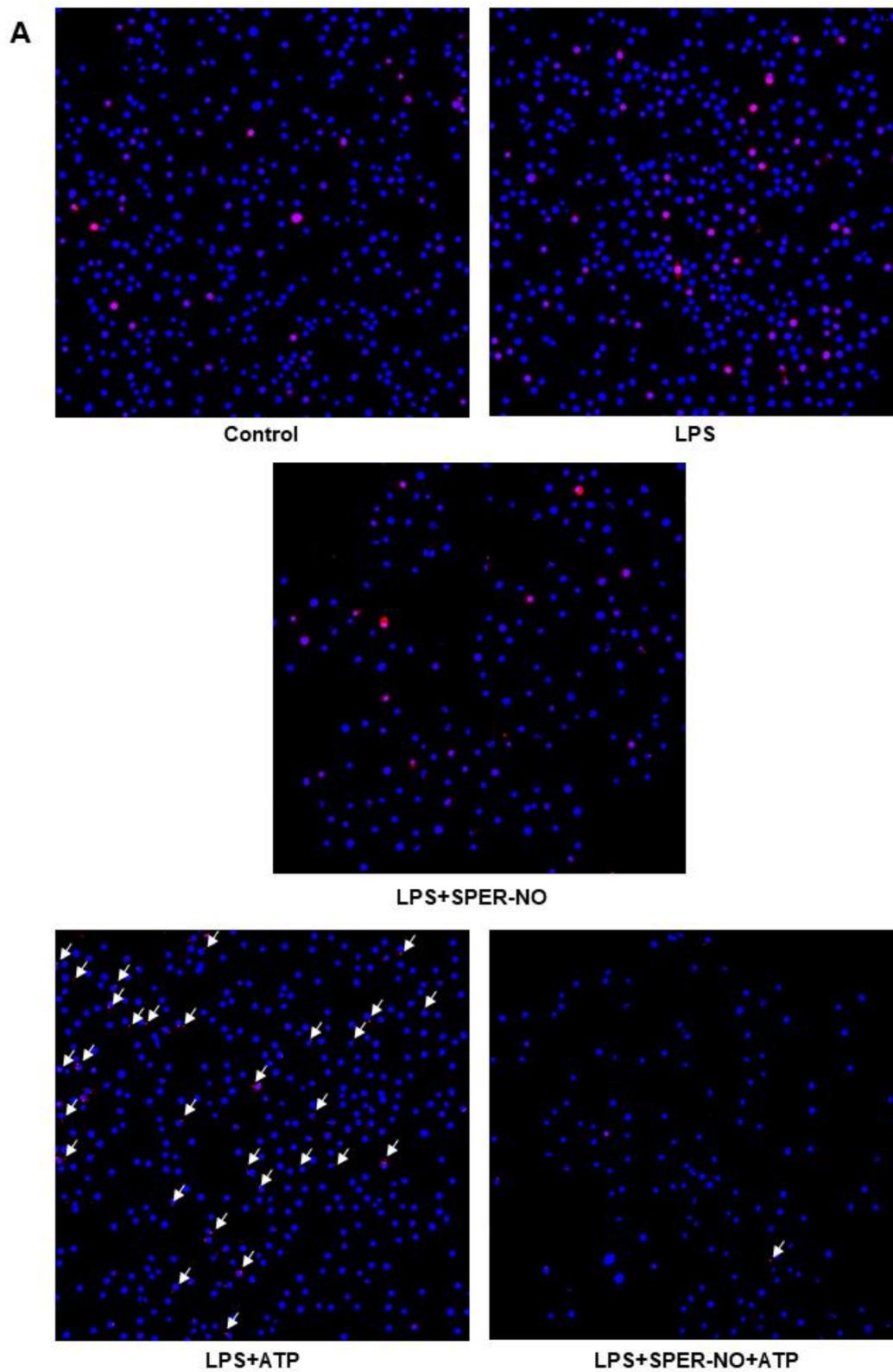
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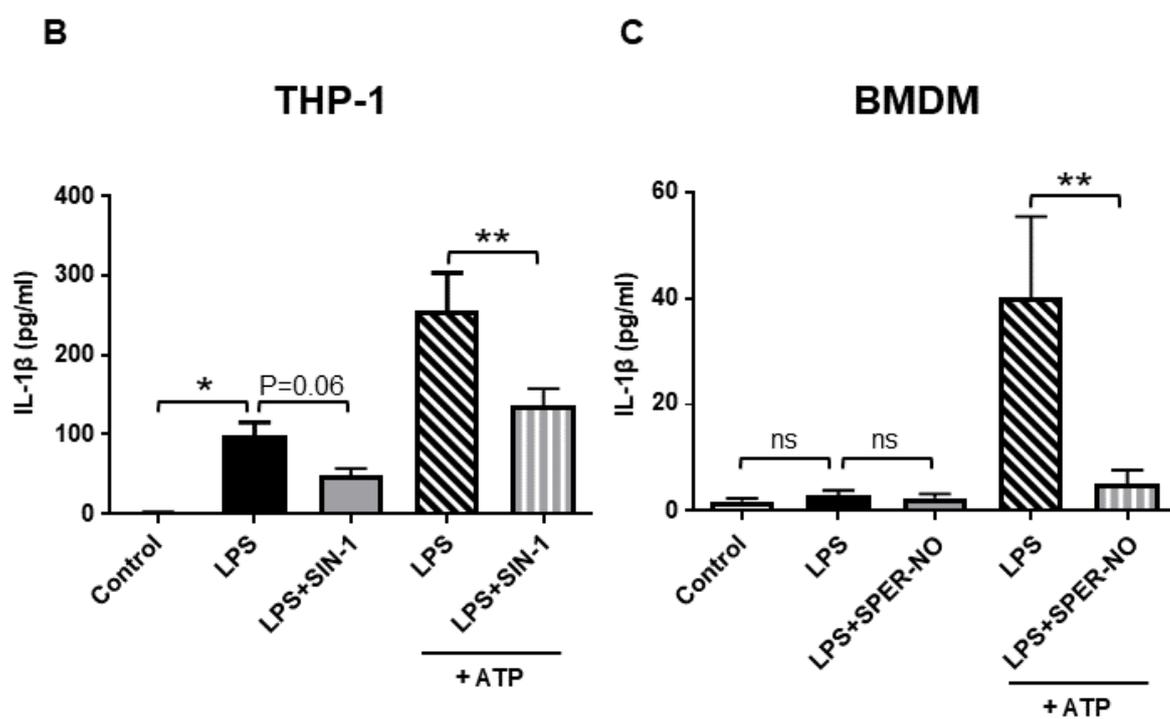
Figure 3

Figure 3: Effect of SIN-1 and SPER-NO on inflammasome assembly and IL-1β production. (A) Immunofluorescence images of ASC-specking in mCerulean tagged ASC immortalised mouse BMDMs stimulated with LPS (1 µg/ml) and/or ATP (3 mM) co-treated with SPER-NO (250 µM) (x20 magnification). IL-1β concentration in the growth medium measured by ELISA following inflammasome activation with LPS (1 µg/ml) and/or ATP (3 mM) and co-treated with either SIN-1 (500 µM) in THP-1 cells (B) or SPER-NO (250 µM) in BMDMs (C). Data are expressed as mean ± SEM (n=4-5 per group). *P<0.05 **P<0.01 for one-way ANOVA followed by Newman Keuls multiple comparisons test.
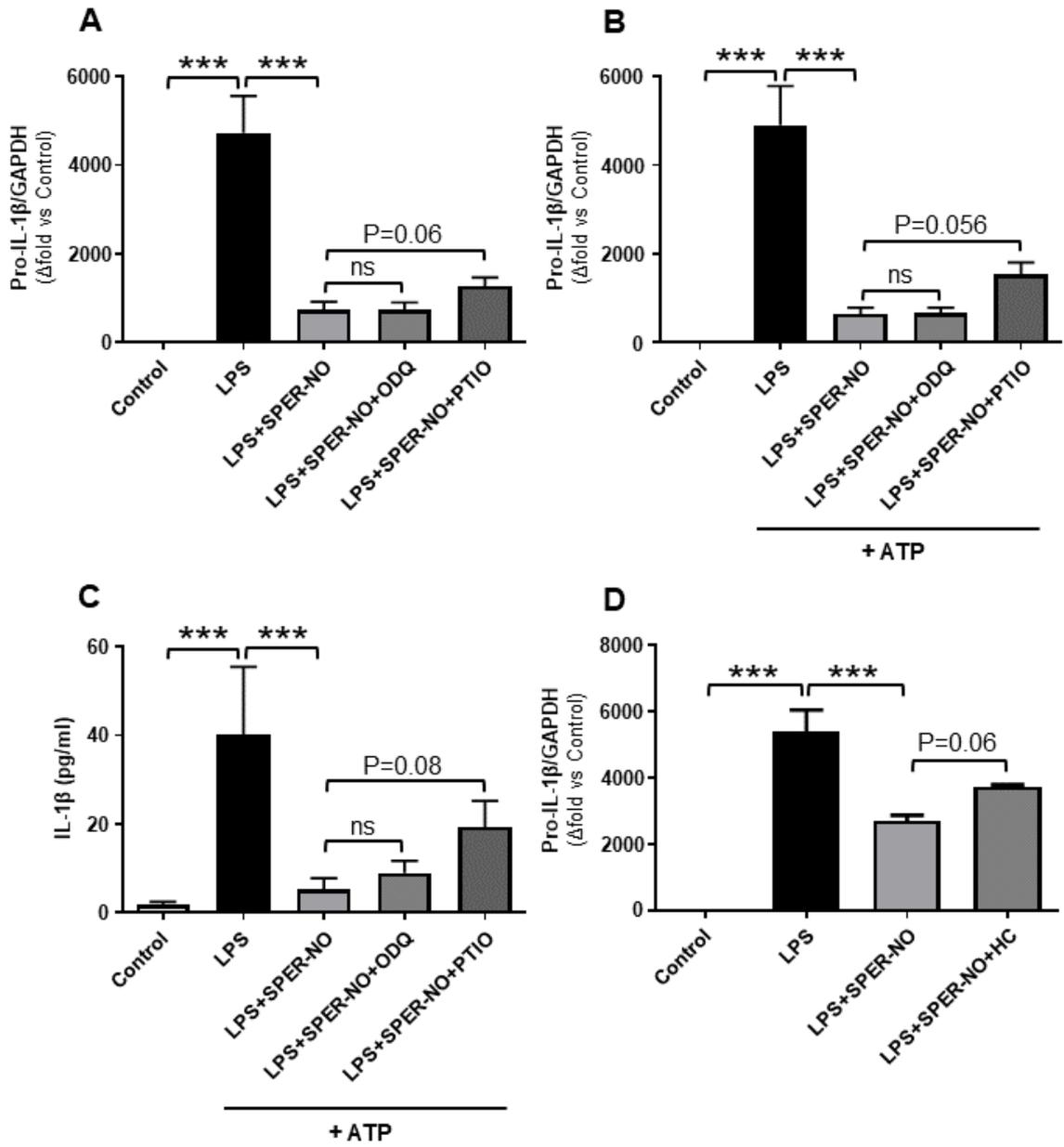
Figure 4

Figure 4: Effect of an sGC inhibitor and NO scavengers on SPER-NO-mediated inhibition of inflammasome priming and activation. The effect of ODQ (10 µM) and carboxy-PTIO (200 µM) on pro-IL-1β mRNA was measured in LPS (1 µg/ml)-stimulated (A) and LPS plus ATP (3 mM)-stimulated (B) mouse immortalised BMDMs using real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. (C) IL-1β protein levels in the growth medium of mouse BMDMs following inflammasome activation with LPS plus ATP and co-treated with ODQ and/or SPER-NO were measured by ELISA. (D) The effect of hydroxocobalamin (100 µM) on SPER-NO-mediated inhibition of LPS-induced upregulation of pro-IL-1β mRNA expression was measured by real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=3-5 per group). ***$P<0.001$ for one-way ANOVA followed by Newman Keuls multiple comparisons test.
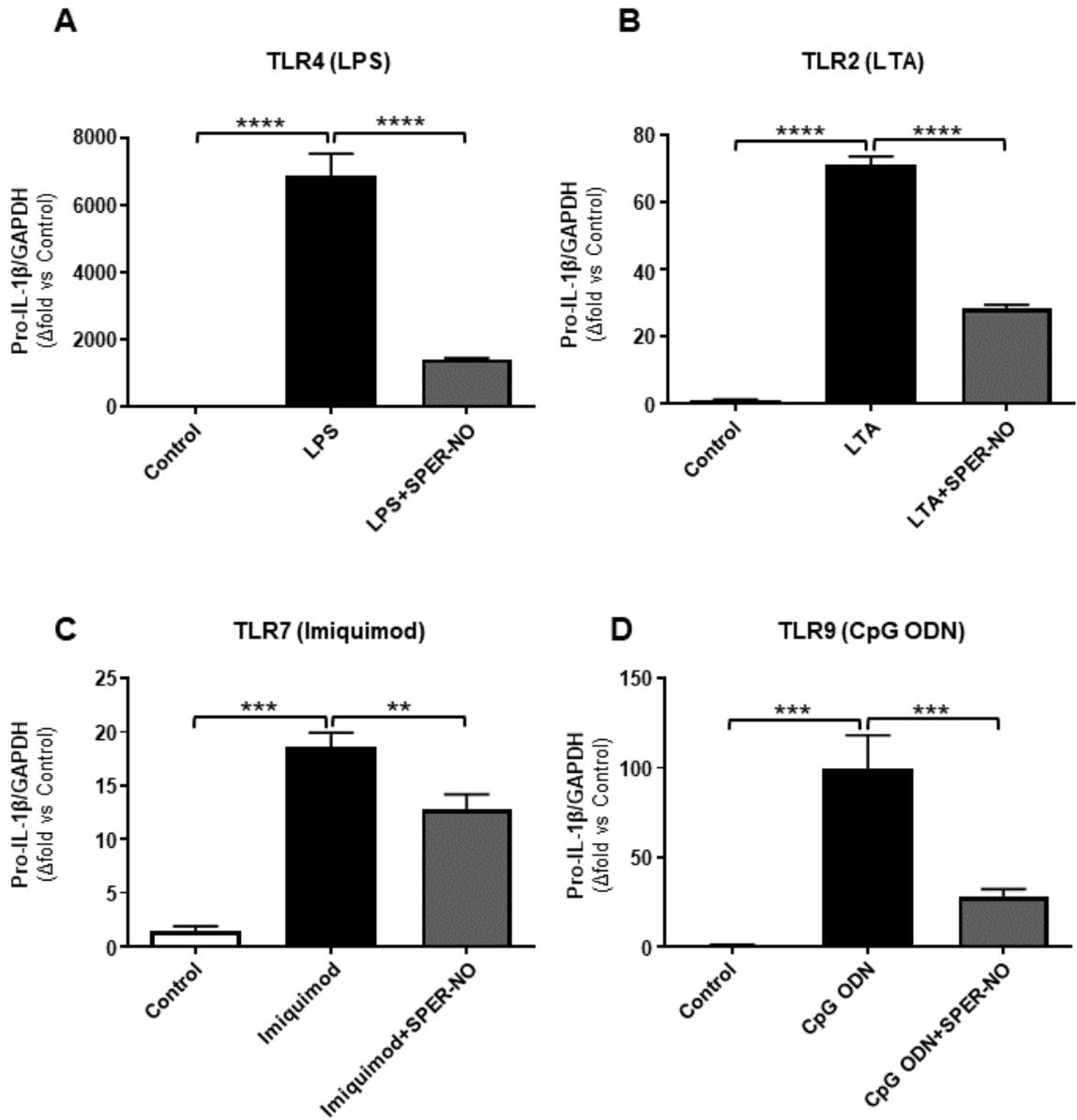
Figure 5

Figure 5: Effect of SPER-NO on inflammasome priming downstream of various TLR subtypes. The effect of SPER-NO on upregulation of pro-IL-1β mRNA in mouse immortalised BMDMs in response to LPS (1 μg/ml) (TLR4 agonist; A) LTA (1 μg/ml) (TLR2 agonist; B), imiquimod (20 μg/ml) (TLR7 agonist; C), and CpG ODN (2 μM) (TLR9 agonist; D) was measured using real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=3-7). **P<0.01 ***P<0.001 ****P<0.0001 for one-way ANOVA followed by Newman Keuls multiple comparisons test.
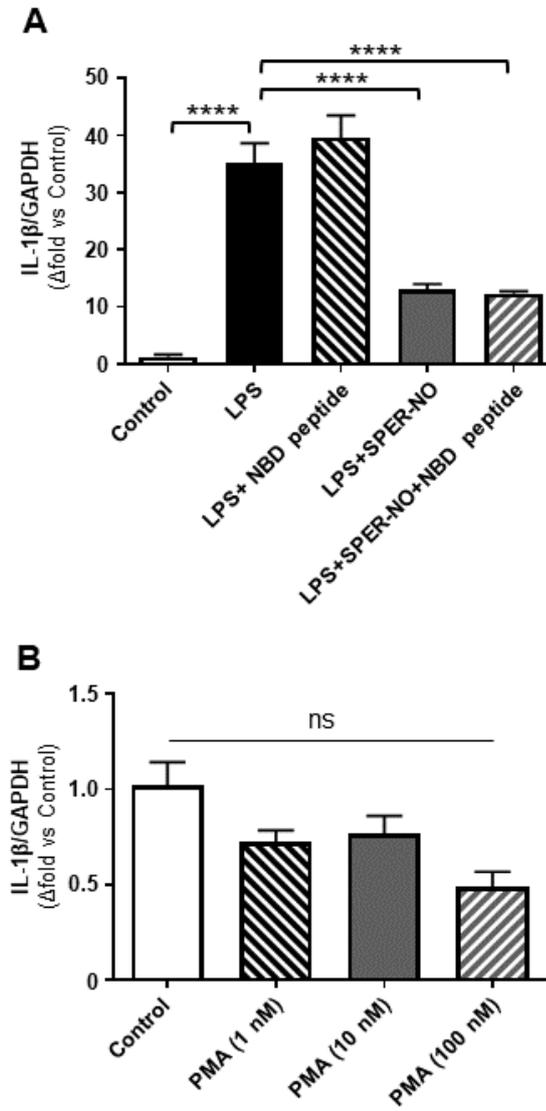
Figure 6

Figure 6: Effect of an NF-κB inhibitor and activator on mRNA expression of pro-IL-1β in immortalised mouse BMDMs. (A) The effect of NBD blocking peptide (25 μM) on pro-IL-1β mRNA expression in LPS stimulated and LPS plus SPER-NO treated BMDMs (B) Pro-IL-1β mRNA expression was measured in BMDMs treated with a range of PMA concentrations (1 nM - 100 nM). mRNA expression was measured using real-time PCR and quantified using the comparative CT method, with GAPDH used as the housekeeping gene. Data are expressed as mean ± SEM (n=4). ****$P<0.0001$ for one-way ANOVA followed by Newman Keuls multiple comparisons test.

Chapter 6:

# Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage and dysfunction in salt-sensitive hypertension

Pharmacological inhibition of the NLRP3 inflammasome reduces blood pressure, renal damage and dysfunction in salt-sensitive hypertension

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Abstract

Aims: Renal inflammation, leading to fibrosis and impaired function is a major contributor to the development of hypertension. The NLRP3 inflammasome mediates inflammation in several chronic diseases by processing the cytokines pro-interleukin (IL)-1β and pro-IL-18. In this study, we investigated whether MCC950, a recently-identified inhibitor of NLRP3 activity, reduces blood pressure (BP), renal inflammation, fibrosis and dysfunction in mice with established hypertension.

Methods and Results: C57BL6/J mice were made hypertensive by uninephrectomy and treatment with deoxycorticosterone acetate (2.4 mg/d, *s.c.*) and 0.9% NaCl in the drinking water (1K/DOCA/salt). Normotensive controls were uninephrectomised and received normal drinking water. 10 days later, mice were treated with MCC950 (10 mg/kg/d, *s.c.*) or vehicle (saline, *s.c.*) for up to 25 days. BP was monitored by tail-cuff or radiotelemetry; renal function by biochemical analysis of 24-hour urine collections; and kidney inflammation/pathology was assessed by real-time PCR for inflammatory gene expression, flow cytometry for leukocyte influx and Picrosirius red histology for collagen. Over the 10 days post-surgery, 1K/DOCA/salt-treated mice became hypertensive, developed impaired renal function, and displayed elevated renal levels of inflammatory markers, collagen and immune cells. MCC950 treatment from day 10 attenuated 1K/DOCA/salt-induced increases in renal expression of inflammasome subunits (NLRP3, ASC, pro-caspase-1) and inflammatory/injury markers (pro-IL-18, pro-IL-1β, IL-17A, TNF-α, osteopontin, ICAM-1, VCAM-1, CCL2, vimentin), each by 25-40%. MCC950 reduced interstitial collagen and accumulation of certain leukocyte subsets in kidneys of 1K/DOCA/salt-treated mice, including CD206^{+} (M2-like) macrophages and interferon-gamma-producing T cells. Finally, MCC950 partially reversed 1K/DOCA/salt-induced elevations in BP, urine output, osmolality, [Na^{+}] and albuminuria (each by 20-30%). None of the above parameters were altered by MCC950 in normotensive mice.

Conclusion: MCC950 was effective at reducing BP and limiting renal inflammation, fibrosis and dysfunction in mice with established hypertension. This study provides proof-of-concept that pharmacological inhibition of the NLRP3 inflammasome is a viable anti-hypertensive strategy.

Keywords: Hypertension; NLRP3 inflammasome; MCC950; renal inflammation; renal fibrosis

Introduction

Through baroreceptor-mediated detection of changes in blood pressure (BP), and the subsequent regulation of Na^+/H_2O re-uptake and blood volume, the kidneys play a major role in BP homeostasis [1,2]. However, chronic exposure to excessive salt, as may occur with a Western diet, can impair the ability of the kidneys to maintain the pressure-natriuresis relationship, resulting in the development of hypertension [3-5]. Although there is still debate around the precise mechanisms by which high salt promotes renal dysfunction, there is a growing body of evidence to suggest that inflammation is likely to be an important factor. In experimental models, salt-sensitive hypertension has been shown to be associated with increased renal expression of pro-inflammatory molecules such as cytokines, chemokines and adhesion molecules [6-8]. This in turn leads to the accumulation of immune cells such as macrophages and T cells in the tubulointerstitium, which promote tissue damage, fibrosis and dysregulation of Na^+ transport [6-11]. Therefore, further understanding of the mechanisms that cause the immune system to become activated during salt-sensitive hypertension may lead to novel pharmacological treatment options and better management of the condition in the clinic.

Inflammasomes are cytosolic signalling complexes that sense danger signals emanating from pathogens or damaged host cells and then respond by initiating an inflammatory cascade [8,12,13]. Of the inflammasomes identified to date, the NLRP3 inflammasome is the best characterized, consisting of a pattern recognition receptor, NLRP3; an adaptor protein, ASC; and the effector molecule, caspase-1 [8,12,14-18]. The NLRP3 inflammasome can recognize a diverse range of pathogen- and host-derived danger-associated molecular patterns (PAMPs and DAMPs, respectively) including bacterial lipopolysaccharides, reactive oxygen species (ROS), and high concentrations of salt and microcrystals [19,20]. Following detection of these PAMPs or DAMPs, the components of the NLRP3 inflammasome oligomerise, resulting in auto-cleavage and activation of caspase-1. Caspase-1 then proteolytically processes pro-IL-1β and pro-IL-18 into their

active forms, which are released from the cell of origin and target neighbouring cells to propagate an inflammatory response [12-14,21].

Inflammasomes are well established to be crucial mediators of inflammation in several chronic inflammatory diseases such as rheumatoid arthritis [22], gout [23,24], Alzheimer's Disease [25] and atherosclerosis [21,26]. More recently, studies by our group and others using a variety of experimental models have shown that the development of hypertension and its associated renal inflammation is at least partially dependent on the presence of a functional NLRP3 inflammasome complex [8,27-29]. This implies that inflammasomes could represent a novel target for future therapies to prevent renal dysfunction and reduce BP in hypertension.

MCC950 is a diarylsulfonylurea-containing compound that was shown *in vitro* to potently (i.e. at nanomolar concentrations) and selectively inhibit the oligomerisation and activation of the NLRP3 inflammasome [30]. Furthermore, Coll *et al.* showed that treatment with MCC950 had protective effects in two experimental models of NLRP3-associated diseases, namely experimental autoimmune encephalitis and Muckle-Wells syndrome [30]. In a pilot study, we provided proof-of-concept that MCC950 was effective at lowering tail cuff BP and renal expression of pro-inflammatory cytokines in mice, even after hypertension was established with the one-kidney, deoxycorticosterone acetate and salt (1K/DOCA/salt) model [8]. Thus, in the present study, we sought to extend these findings and gain insight into the physiological processes underlying the anti-hypertensive effects of MCC950 by examining its effects on additional hemodynamic parameters (e.g. mean arterial pressure, diastolic BP, heart rate), immune cell activation, renal pathology and function.

Methods

<u>Animals</u>

Male C57BL/6J mice, aged 10-12 weeks and weighing 25-30 g were used in this study. Mice were obtained from Monash Animal Services (Australia) and housed in OptiMice® cages (Animal Care Systems, USA) with *ad libitum* access to standard chow and drinking water. All procedures on mice were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and were approved by the Monash University Animal Research Platform Animal Ethics Committee (Ethics number: MARP/2013/043).

<u>Induction of hypertension and treatment of mice with MCC950</u>

A salt-sensitive model of hypertension was used in this study, wherein mice were uninephrectomized and treated with deoxycorticosterone acetate (DOCA) and salt. Mice were placed under general anaesthesia by inhalation of 2% isoflurane. A left-flank incision of the skin and muscle layers was made allowing ligation of the renal artery and excision of the left kidney. The muscle layer was sutured and, prior to closing the skin, a subcutaneous pouch was made beginning at the site of the wound and extending towards the scapular region. A slow-release DOCA pellet (2.4 mg/d, *s.c.*; Innovative Research of America, USA) was implanted into this pouch and the skin was then sutured. Mice were allowed to recover prior to being returned to their home cages where they were provided with *ad libitum* access to 0.9% saline instead of normal drinking water (1K/DOCA/salt). Normotensive control mice were uninephrectomized and maintained on normal drinking water post-surgery (1K/placebo).

Ten days after the induction of hypertension, mice were randomly assigned to commence treatment for up to 28 days with either the novel NLRP3 inflammasome inhibitor, MCC950 (10 mg/kg/d, *s.c.*) or vehicle (0.9% saline, 0.5 µL/h, *s.c.*) via implantation of a micro-osmotic

minipump (Alzet, USA). Again, mice were placed under general anesthesia by inhalation of 2% isoflurane. A small incision was made at the nape of the neck, and blunt dissection was used to make a subcutaneous pouch along the right side of the mouse. The minipump containing either MCC950 or saline was implanted into the pouch, and the incision was closed using a wound clip. In pilot studies (n=3-4 per group), the effects of 11 d treatments with MCC950 doses of 2, 5 and 10 mg/kg/d on BP in mice with established 1K/DOCA/salt-induced hypertension were examined (data not shown). The 10 mg/kg/d dose was found to be the only one that was effective at reversing BP and hence was chosen for further investigation in the present study. This is equivalent to the MCC950 dose used to inhibit NLRP3-mediated processes in mice in previous studies [31,32].

Monitoring of blood pressure

BP was monitored either via tail-cuff plethysmography or radiotelemetry. Tail-cuff plethysmography was carried out on a MC4000 Multichannel System (Hatteras Instruments, USA). All mice underwent daily training on the tail cuff device for at least 3 days prior to induction of hypertension. Mice then had their BP recorded just prior to surgery (day 0), and again on days 3, 7, 10, 14, 17 and 21 post-surgery. In a separate cohort of mice, BP was monitored continuously using radiotelemetry. Mice were implanted with a telemeter probe (Model TA11PA-C10, Data Sciences International, USA) and allowed 10 days to recover from surgery. The probe was then switched on to obtain baseline recordings of systolic BP, diastolic BP, mean arterial pressure (MAP) and heart rate for 3 consecutive days. Mice then underwent surgery to induce hypertension as described above, and the same parameters were recorded every 10 min throughout the remainder of the treatment period. Data is expressed as an average of measurements collected over a 3 day interval.

Gene expression in the kidney

Following treatment, mice were killed via inhalation of CO_2 and perfused through the left ventricle with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM

Na_2HPO_4 and 1.47 mM KH_2PO_4; pH 7.4) containing 0.2% Clexane (400 IU; Sanofi Aventis, France). The right kidney was excised, and cut in half along the coronal plane. One half of the kidney was used immediately for flow cytometric analysis, while the other half was either snap-frozen in liquid N_2 or fixed in 10% formalin and stored at -80°C for subsequent RNA extraction or -20°C for immunohistochemistry, respectively. Upon removal from -80°C storage, kidney halves were further cut transversely with one of the quarters used for RNA extraction using the RNeasy Mini Kit (Qiagen, Germany). The yield and purity of the RNA was determined using QiaExpert (Qiagen, Germany). RNA was reverse transcribed to cDNA using a reverse transcription kit (Applied Biosystems, USA). The cDNA was then used as a template in real-time PCR using commercially available predesigned TaqMan® primer/probe sets (Life Technologies, USA) to measure mRNA expression levels of the inflammasome subunits NLRP3, ASC and pro-caspase-1; the pro-inflammatory molecules pro-IL-1β, pro-IL-18, IL-6, IL-17A, osteopontin (SPP1), TNFα, ICAM-1, VCAM-1, and CCL2; the alpha1 subunits of collagen Type I, III, IV and V; TGF-β; and vimentin, a marker of epithelial-mesenchymal transition (EMT). GAPDH was used as a house-keeping gene. Real-time PCR was performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) and the comparative Ct method was used to calculate the fold-change in mRNA expression relative to the control samples i.e. 1K/placebo + vehicle-treated mice [33].

Flow cytometry

Flow cytometry was performed on cell suspensions derived from freshly isolated kidney halves. Briefly, one half of the right kidney was digested mechanically using scissors and then enzymatically by incubation for 45 min at 37°C in 1 mL of digestion buffer comprising collagenase type XI (125 U/mL), collagenase type I-S (450 U/mL) and hyaluronidase (450 U/mL) dissolved in sterile PBS with calcium and magnesium (Sigma, USA). The digested tissue was then passed through a 70 μm sterile filter (BD Biosciences, USA) and the cells were pelleted by centrifugation at 1200 RPM for 10 min. The cell pellet was re-suspended

in 3 mL of 30% Percoll™ solution (GE Healthcare Life Science, UK), carefully under-laid with 3 mL of 70% Percoll™ solution and spun at 2500 RPM at 25°C for 25 min with the brakes of the centrifuge turned off. The layer containing mononuclear cells – lying between the Percoll™ gradients – was collected and re-suspended in sterile Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma, USA) and stored at 4°C until staining. Cells were then stained at 4°C for 15 min first with the Live/Dead Aqua Stain (Life Technologies, USA) followed by the antibody cocktail listed in Table 1 (Online Supplementary File). Finally, stained cells were re-suspended in flow cytometry buffer (1% bovine serum albumin in PBS) containing 1% formalin. For detection of IFN-γ-producing renal T cells, prior to staining, kidney mononuclear cells were stimulated with phorbol myristate acetate (PMA; 50 ng/mL) and ionomycin (20 ng/mL) for 5 h in the presence of Golgiplug/Golgistop (BD Biosciences, USA). Following stimulation, cells were washed in PBS and live/dead and surface marker staining was performed as described above. Following surface staining, cells were fixed and permeabilised using a fix/permeabilisation solution (eBioscience, USA) for 30 min and then washed with a permeabilisation wash buffer. Cells were then incubated with an anti-IFN-γ antibody at room temperature for 20 min and then washed and resuspended in FACS buffer containing 1% formalin. All samples were analysed using a Fortessa X-20 instrument controlled by FlowDiva software (BD Biosciences). Data were analysed using FlowJo software v10 (FlowJo, USA; see Supplementary Material for the gating strategy).

<u>Picrosirius red staining for collagen</u>

Kidney halves were placed in 10% formalin for 3 days, following which they were embedded in paraffin and cut into 10 µm sections. Following hydration by immersion in xylene and a series of graded ethanol solutions, sections were incubated with Celestine blue to stain for nuclei, and then counterstained with hematoxylin (Amber Scientific, Australia) and 0.3% Picrosirius red solution (Polysciences Inc, USA). The sections were mounted in DPX solution (Ajax Finechem, Australia) and imaged at 10x and 40x magnification using a bright-field microscope (Leica Biosystems, Germany) and 20x magnification using a polarised

microscope (Olympus, Japan). ImageJ software was used to analyse collagen content by calculating the area of collagen fibres (as percentage of the total area per field of view) in 14 randomly-selected fields-of-view on 2 serial sections. Assessment of collagen content was performed by an investigator who was blinded to the *in vivo* treatment corresponding to each sample.

Assessment of kidney function using metabolic cages

Mice were housed individually in mouse-specific metabolic cages (Scientific Glassware, Faculty of Medicine, University of Melbourne) for 24 h intervals, allowing the volume of water/saline intake and urine production over that time to be accurately measured. Prior to taking any measurements, all mice were habituated by placing them in the metabolic cages for 8 h once and then for 24 h on two further occasions. Three separate measurements were then obtained across the 1K/DOCA/salt plus MCC950 or vehicle treatment period including: on day -1 to obtain baseline parameters; on day 9 to assess the impact of 1K/DOCA/salt treatment on kidney function, and finally on day 20 to assess the impact of MCC950 versus vehicle treatment. Once volume measurements were obtained, the collected urine was stored at -80°C for analysis of osmolality (Advanced Osmometer 2020; Advanced Instruments, USA), Na^+ concentration (RAPIDChem744, Siemens, Germany) and albuminuria (Albuwell M, Exocell, USA).

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Data collected from metabolic cage studies were analysed by Student's unpaired t-test. BP and heart rate were analysed by two-way repeated measures analysis of variance (ANOVA) with a Newman-Keul's *post hoc* test. All other data were analysed by two-way analysis of variance (ANOVA) with Newman-Keul's *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). All statistical analyses were performed using GraphPad

Prism Software v6.04 (USA).

Results

<u>Intervention with MCC950 reduces BP in mice with established hypertension</u>

Consistent with our previous report [8], 1K/DOCA/salt treatment in mice caused a rapid increase in systolic BP (measured by tail cuff) which reached a plateau of approximately 30-35 mmHg above baseline within 10 days post-surgery (Fig 1A). In mice that were subsequently treated with the vehicle for MCC950, systolic BP continued to rise gradually over the following 11 days (Fig 1A). By contrast, in mice that received 10 mg/kg/d of MCC950, systolic BP gradually decreased such that after 11 days, BP was ~20 mmHg lower in MCC950- than in vehicle-treated animals (Fig 1A). A common consequence of high BP is cardiac hypertrophy [34]. 1K/DOCA/salt-induced hypertension was found to increase the ratio of heart weight to body weight compared to normotensive mice (Fig 1B). Importantly, hypertensive mice treated with MCC950 displayed blunted cardiac hypertrophy (Fig 1B). In normotensive animals, systolic BP remained unchanged during the first 10 days after surgery, and both systolic BP and heart weight to body weight ratio were not further altered by MCC950 (Fig 1A-B).

Radiotelemetry was performed in a subset of mice to further characterise the effects of MCC950 on hemodynamic parameters during 1K/DOCA/salt-dependent hypertension. Similar to that observed using tail cuff plethysmography, 1K/DOCA/salt treatment caused a 30-40 mmHg increase in systolic BP over the first 10 days (Fig 1C). Similar increases were observed for both diastolic BP and MAP (Fig 1D-E). Also consistent with findings from tail cuff studies, the MCC950 intervention afforded protection against 1K/DOCA/salt-induced hypertension, such that by the end of the 28 day treatment period, all three BP parameters (i.e. systolic BP, diastolic BP and MAP) were ~10-12 mmHg lower in MCC950- versus vehicle-treated mice (Fig 1C-E). Heart rate was not significantly different between the MCC950- and vehicle-treated groups, before or after the drug intervention period (Fig 1F).

MCC950 reduces expression of inflammatory markers and leukocyte infiltration in kidneys of 1K/DOCA/salt-treated mice

Real-time PCR analysis revealed that 1K/DOCA/salt-induced hypertension was associated with increased renal mRNA expression levels of NLRP3, ASC, pro-caspase-1, pro-IL-1β and pro-IL-18 (Fig 2A-E), confirming that this model of hypertension is associated with priming of the inflammasome/IL-1β/IL-18 signalling system in the kidneys. Likewise, increases in expression of several additional pro-inflammatory genes were also observed including the cytokines IL-6, IL-17A, TNF-α and IL-12 (Fig 3A-D); the adhesion molecules, ICAM-1 and VCAM-1 (Fig 3E-F); the chemokine, CCL2 (Fig 3G); and a marker of tubular damage, osteopontin (Fig 3H). Treatment of hypertensive mice with MCC950 reduced the expression of most of these genes by 25-40% (Fig 2 and 3), with the exceptions being IL-6 (for which the trend towards a 30% reduction was not statistically significant) and IL-12. Notably, MCC950 had no effect on basal levels of expression of any of these genes in normotensive mice (Fig 2 and 3).

Chemokines and adhesion molecules are important mediators of leukocyte trafficking from the circulation into tissues. Consistent with its effects on CCL2, ICAM-1 and VCAM-1 expression, 1K/DOCA/salt-treatment caused an accumulation of leukocytes in the kidney (Fig 4A). This included an increase in CD3$^+$ T cells, and in particular the CD4$^+$ subset, with no significant change in CD8$^+$ T cells (Fig 4B-D). Previous studies have shown that IL-1β and IL-18 can act in concert with IL-12 to promote the production of the T cell-derived pro-inflammatory cytokine interferon-gamma (IFNγ) [35]. Given that expression of IL-1β, IL-18 and IL-12 were all upregulated in kidneys of mice with 1K/DOCA/salt-induced hypertension, we investigated whether there might also be an increase in T cell-dependent production of IFNγ. Although, overall expression levels of IFN-γ were not significantly altered in whole kidney homogenates from 1K/DOCA/salt-treated mice (data not shown), there was a 3-fold increase in IFN-γ-producing T cells (Fig 4E). Of note, MCC950 markedly inhibited the

accumulation of total leukocytes and T cells in the kidneys of 1K/DOCA/salt-treated mice (Fig 4A-D), including those that produced IFN-γ (Fig 4E).

In addition to the accumulation of T cells, 1K/DOCA/salt-induced hypertension in mice was associated with marked increases in numbers of myeloid lineage cells (CD45$^+$CD11b$^+$) and macrophages (CD45$^+$CD11b$^+$F4/80$^+$) in the kidneys, with further analysis of the macrophage subsets revealing that there was a significant increase in the "M2"-(F4/80$^+$CD206$^+$) but not the "M1"-like (F4/80$^+$CD206$^-$) phenotype (Fig 5A-D). Treatment of 1K/DOCA/salt hypertensive mice with MCC950 mice reduced total myeloid cell and macrophage numbers in the kidneys (Fig 5A-B). More specifically, MCC950 treatment appeared to have its largest effects on the M2-like macrophage population (Fig 5C). Again, MCC950 appeared to have no effect on numbers of any leukocyte subsets in the kidneys of normotensive mice (Fig 4 & 5).

<u>MCC950 reduces the accumulation of collagen in the kidneys of 1K/DOCA/salt-treated mice</u>

Kidney sections from 1K/DOCA/salt-treated mice displayed a ~3-fold increase in renal interstitial collagen protein expression (whether assessed by bright field or polarised microscopy) compared to normotensive mice (Fig 6A and B). Treatment with MCC950 reduced collagen deposition by ~30% in mice with 1K/DOCA/salt-induced hypertension but had no effect on the amount of collagen protein present in the kidneys of normotensive mice (Fig 6A and 6B).

The increase in collagen protein in kidneys of 1K/DOCA/salt-treated mice was reflected at the gene level with mRNA expression of four of the predominant renal collagen subtypes (I, III, IV and V) elevated compared to kidneys from 1K/placebo-treated mice (Fig 6C-F). Renal mRNA expression of the pro-fibrotic cytokine, TGF-β, was also significantly increased by 1K/DOCA/salt-treatment (Fig 6G). Treatment with MCC950 reduced mRNA expression of TGF-β along with collagen III, IV and V (Fig 6C-G). 1K/DOCA/salt hypertension was

associated with EMT, as evidenced by an increase in expression of vimentin in the kidneys, and this was also abrogated by ~50% by MCC950 treatment.

MCC950 improves kidney function in 1K/DOCA/salt-treated mice

Inflammation and fibrosis of the kidneys are associated with impaired function and a shift in the pressure-natriuresis relationship. Metabolic cage studies were performed to assess the impact of 1K/DOCA/salt-induced hypertension on kidney function, and to determine if treatment with MCC950 protects against functional impairment. Ten days of 1K/DOCA/salt treatment resulted in a marked increase in the volume of urine excreted (11-fold) and in the amount of saline consumed (5-fold; Fig 7A-B). The osmolality of the urine was ~3.5 times higher following 10 days of 1K/DOCA/salt-treatment than it was prior to the induction of hypertension, including a 27-fold increase in urinary [Na^+] (Fig 7C-D). Excessive amounts of albumin leakage into the urine is a key sign of kidney dysfunction, and in mice treated with 1K/DOCA/salt it was found that albuminuria levels were increased by 18-fold (Fig 8E). In hypertensive mice that were subsequently treated with vehicle for a further 11 days, these parameters remained unchanged (Fig 7A-E). By contrast, MCC950-treament reduced urine volume, saline intake, urine osmolality, urine [Na^+] and albuminuria, such that after 11 days all of these parameters were lower than in vehicle-treated animals (Fig 7A-E).

Discussion

The major new findings from this study are that MCC950, a selective small-molecule NLRP3 inflammasome inhibitor, is highly effective at reducing renal inflammation and fibrosis, and improving renal function, even when administered 10 days after the establishment of 1K/DOCA/salt-induced hypertension in mice. Moreover, these protective effects of MCC950 on the kidneys were associated with a modest reduction in BP and blunted cardiac hypertrophy. Hence, together with earlier reports of BP-lowering and renal anti-inflammatory effects of ASC-deficiency and IL-1R antagonism [6,8], this study highlights the NLRP3 inflammasome as a promising target for therapies aimed at reducing the end-organ damage associated with hypertension.

It is well established that hypertension is associated with increased expression of adhesion molecules and pro-inflammatory cytokines, and the accumulation of inflammatory T cells and macrophages in the kidneys [6-8]. Moreover, these inflammatory events are thought to contribute to the renal fibrosis and damage that results in disruption of the pressure-natriuresis relationship, and the re-setting of BP at a chronically elevated level [7-11,13,37]. Using transgenic mouse models, we and others have shown that NLRP3 inflammasome activity is essential for the development of renal inflammation and elevated BP in response to a variety of hypertensive stimuli including 1K/DOCA/salt, angiotensin II and renal artery clipping [8,27,29]. While these findings implied that the NLRP3 inflammasome is a promising target for future anti-hypertensive therapies, it remained to be determined (in a more clinically relevant context) whether inflammasome inhibition could *reverse* BP and *reduce* markers of renal inflammation and dysfunction when hypertension is already established. Indeed, here we show that administration of MCC950 to mice, 10 days after the induction of hypertension with 1K/DOCA/salt (i.e. when BP is at its highest), is profoundly effective at limiting renal inflammation as assessed by marked reductions in inflammasome priming, expression of adhesion molecules, chemokines and pro-inflammatory cytokines, and the accumulation of T cells and macrophages. Intervention with

MCC950 was also highly effective at suppressing renal interstitial collagen deposition and levels of the pro-fibrotic cytokine, TGF-β. Importantly, these effects culminated in significant improvements in renal function, both in terms of Na$^+$ and electrolyte handling, and also less albumin leakage into the urine. Hence, our findings provide proof-of-concept that pharmacological modulation of inflammasome activity may be a viable therapeutic strategy for limiting renal damage, even in patients with existing disease.

Macrophages are a major source of TGF-β and as such are important contributors to fibrosis in a number of disease settings [38,39]. Although we did not examine directly whether macrophages in the kidneys of hypertensive mice were a source of TGF-β, we did show that the inhibitory effects of MCC950 on 1K/DOCA/salt-dependent macrophage infiltration of the kidneys occurred in parallel with reductions in both TGF-β and collagen mRNA expression. As for the macrophage subtype that was likely involved, we showed that 1K/DOCA/salt-induced hypertension was associated with a marked increase in accumulation of CD206$^+$ (M2-like) macrophages in the kidneys and that MCC950 treatment markedly attenuated this. By contrast, numbers of CD206$^-$ (M1-like) macrophages were not different between normotensive and hypertensive mice and were unaffected by MCC950 treatment. M2 macrophages have been shown to play an important role in promoting fibrosis of other tissues; for example, in the heart after myocardial infarction [40], the lungs after bleomycin treatment [41], and the skin in models of experimental scleroderma [42]. Furthermore, we have shown that M2-like macrophages are major contributors to aortic fibrosis in an alternative model of hypertension, namely that induced by angiotensin II infusion [34]. The present findings suggest that M2 macrophages are also potentially important mediators of renal fibrosis in the setting of 1K/DOCA/salt-induced hypertension and we speculate that the protective effect of MCC950 may be at least partly due to its ability to limit the accumulation of these cells. However, it must be acknowledged that macrophages are not the only source of TGF-β as tubular epithelial cells (TECs) and myofibroblasts are also an important source during renal fibrosis [43]. TECs are known to

express components of the inflammasome [44], and thus MCC950 may also act to suppress inflammasome activity in these cells to prevent renal inflammation and fibrosis that is associated with hypertension. Future studies will aim to determine the cellular targets of MCC950.

In addition to increasing macrophage numbers in the kidneys, 1K/DOCA/salt promoted the accumulation of CD4^{+} T cells. Furthermore, T cells were shown to be a source of the pro-inflammatory cytokine IFN-γ. Previous studies have shown that IL-18 can work in concert with IL-12 to promote the production of IFN-γ by T cells [45]. Indeed, we found that both of these cytokines were increased in the kidneys of 1K/DOCA/salt-treated mice, and that MCC950 not only reduced expression of IL-18, but also the accumulation of IFN-γ-producing T cells. Mice deficient in IFN-γ, either through genetic mutation or treatment with an IFN-γ-neutralising antibody, are protected from angiotensin II-dependent hypertension and the associated renal inflammation [46]. Kamat *et al* suggested that one of the mechanisms by which IFN-γ promotes hypertension is by increasing the abundance of the phosphorylated forms of the Na-K-2Cl cotransporter, Na-Cl cotransporter, and Ste20/SPS-1-related proline-alanine-rich kinase in tubular epithelial cells, thereby increasing Na$^+$ reuptake [46]. Hence, our findings imply that IFN-γ may be a common mediator of renal damage in hypertension irrespective of the stimulus and moreover, that pharmacological inhibition of the NLRP3 inflammasome is an effective means of reversing the activation of this pro-hypertensive pathway.

A novel finding in the present study was that MCC950 partially reversed albuminuria in 1K/DOCA/salt-treated mice, suggesting that it improved glomerular filtration. Curiously, while the intervention with MCC950 reduced urine output, osmolality and [Na$^+$], it also reduced Na$^+$/H$_2$O intake. This highlights a limitation of the current model in that salt was provided to mice *ad libitum* via the drinking water making it difficult to interpret whether the reduced urine output drove the reduced saline intake, or vice versa. A future alternative

approach to directly investigate if MCC950 alters Na$^+$/electrolyte handling by the kidneys might involve challenging treated and untreated animals with equal amounts of salt and volume via an intraperitoneal bolus of salt and water.

The intervention with MCC950 also reduced BP in hypertensive mice by ~10-20 mmHg (i.e. 25% of the 1K/DOCA/salt-induced pressor effect). This was true for systolic, diastolic and, by extrapolation, MAP. In humans, the level of cardiovascular risk halves with every 10 mmHg reduction in diastolic BP [47]. Hence, while the current findings clearly are of potential clinical relevance, compared to its profound effects on renal inflammation and fibrosis, the anti-hypertensive actions of MCC950 were relatively modest. This suggests that there may be some degree of disconnect between renal inflammatory status and BP following 1K/DOCA/salt-treatment; a conclusion that is consistent with previous findings in this and other experimental models of hypertension. For example, Liang et al demonstrated that genetic deficiency of the chemokine, CXCL16, in 1K/DOCA/salt-treated mice afforded marked protection against renal fibrosis, albuminuria, and macrophage and T cell infiltration, yet failed to limit increases in BP [48]. Conversely, using an intervention protocol similar to that used here, we recently demonstrated that the interleukin-1 receptor antagonist (IL-1Ra), anakinra, was comparable to MCC950 in its ability to reduce 1K/DOCA/salt-induced elevations in BP, yet had little impact on expression of renal inflammatory markers and leukocyte accumulation [36]. A potential unifying explanation for these seemingly incongruous findings is that the two major inflammasome-derived cytokines - IL-1β and IL-18 - may play unique roles in the pathophysiology of hypertension, with one acting outside the kidneys to contribute to BP changes, and the other being the more important mediator of renal inflammation and injury. Indeed, in support of a predominantly extra-renal role, IL-1β has been shown to increase endothelial superoxide production and impair endothelium-dependent relaxation in resistance-like arteries [49]. Moreover, IL-1R$^{-/-}$ mice were afforded complete protection against the endothelial dysfunction that normally accompanies chronic aldosterone administration in wild type mice [50], whereas IL-1$\beta^{-/-}$ mice showed no

protection against the acute renal damage caused by ischemia [51]. Thus, future studies aimed at directly comparing the impact of inhibition of IL-1β versus IL-18 on 1K/DOCA/salt-induced hypertension and their respective contributions to renal and non-renal pathologies are certainly warranted.

Currently, the exact mechanism of NLRP3 activation during hypertension remains unknown. Three distinct models which have been extensively studied and proposed to account for NLRP3 activation include: potassium efflux, the generation of ROS and phagolysosomal destabilization leading to cathepsin leakage into the cytosol (as reviewed He, *et al* [52]). While MCC950 is known to selectively inhibit the oligomerisation and activation of the NLRP3 inflammasome [30], it does not influence potassium efflux and calcium flux [30]. Moreover, MCC950 does not directly inhibit sulfonylurea receptor (SUR1, SUR2a and SUR2b)-mediated regulation of $K_{ir6.2}$ currents in recombinant cell lines [53]. Furthermore, there is indirect evidence that MCC950-mediated inhibition of the inflammasome is not via ROS scavenging. ROS is not associated with NLRP3 activation via nigericin and ATP [54,55], which are both potently inhibited by MCC950. The direct mechanism by which MCC950 inhibits inflammasome activity in our model of hypertension and renal inflammation was not the focus of the current study. However, consistent with our previous study that reported MCC950-mediated reversal of 1K/DOCA/salt-induced hypertension and renal inflammatory gene expression, we have now demonstrated that chronic MCC950-treatment significantly reduced inflammasome priming and activation in the 1K/DOCA/salt model of hypertension, which is associated with reductions in BP, renal leukocyte recruitment and fibrosis, and improved renal function.

Regarding the stimuli that cause inflammasome activation in hypertension, there is evidence that both salt-induced interstitial hyperosmolality and aldosterone can act as danger signals, both of which are key components of the 1K/DOCA/salt model. For example, Ip *et al.* showed that hyperosmotic stress induced by high [Na⁺] is detected as a danger signal by cultured macrophages and results in oligomerization of both NLRP3 and

NLRC4 inflammasomes, caspase-1 activation and IL-1β production [56]. Kadoya *et al.* showed that direct stimulation of macrophages *in vitro* with aldosterone also induces caspase-1 activation and IL-18 production, and that these effects were attenuated by eplerenone, an antagonist of the mineralocorticoid receptor [28]. Interestingly, recent evidence suggests that mineralocorticoid receptor antagonists, including eplerenone and spironolactone, are effective in the treatment of many patients with resistant hypertension [57]. Thus, it would be interesting to determine what role inflammasome inhibition plays in these beneficial effects of mineralocorticoid receptor antagonists, and furthermore, whether drugs that directly target the inflammasome (e.g. MCC950) or its cytokine products may be effective alternative therapies for the treatment of resistant hypertension in the clinic. On this latter point, canakinumab, a therapeutic monoclonal antibody against IL-1β, reduced cardiovascular events by 15% in patients with previous myocardial infarction and evidence of systemic inflammation [58]. Although the protective effects of canakinumab occurred independently of any actions on plasma lipids, it was not reported whether IL-1β inhibition lowered BP in these patients. Hence, it will be interesting to see if this was the case and whether an anti-hypertensive action of canakinumab may in part explain its ability to attenuate the risk of cardiovascular events in high risk patients.

In conclusion, this study demonstrates that a novel NLRP3 inflammasome inhibitor, MCC950, protects mice from the renal dysfunction, fibrosis and inflammation associated with the development of 1K/DOCA/salt-induced hypertension. Although several questions remain about the mechanisms involved in this protective effect of MCC950, our findings support the concept that drugs targeting inflammasome activity hold potential as novel therapeutics for the treatment of hypertension and renal damage. Indeed, the favourable pharmacodynamic (IC_{50} for NLRP3 <10 nM) and pharmacokinetic (oral bioavailability of 68%) [30] properties of MCC950 highlight it as a promising lead for the development of such drugs.

CONFLICTS OF INTEREST

MAC currently holds a fractional Professorial Research Fellow appointment at the University of Queensland with his remaining time as CEO of Inflazome Ltd., a company headquartered in Dublin, Ireland that is developing drugs to address clinical unmet needs in inflammatory disease by targeting the inflammasome. EL is a shareholder in IFM Therapeutics, a company that works to improve the lives of patients with serious diseases by developing medicines that target the innate immune system.

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Figures
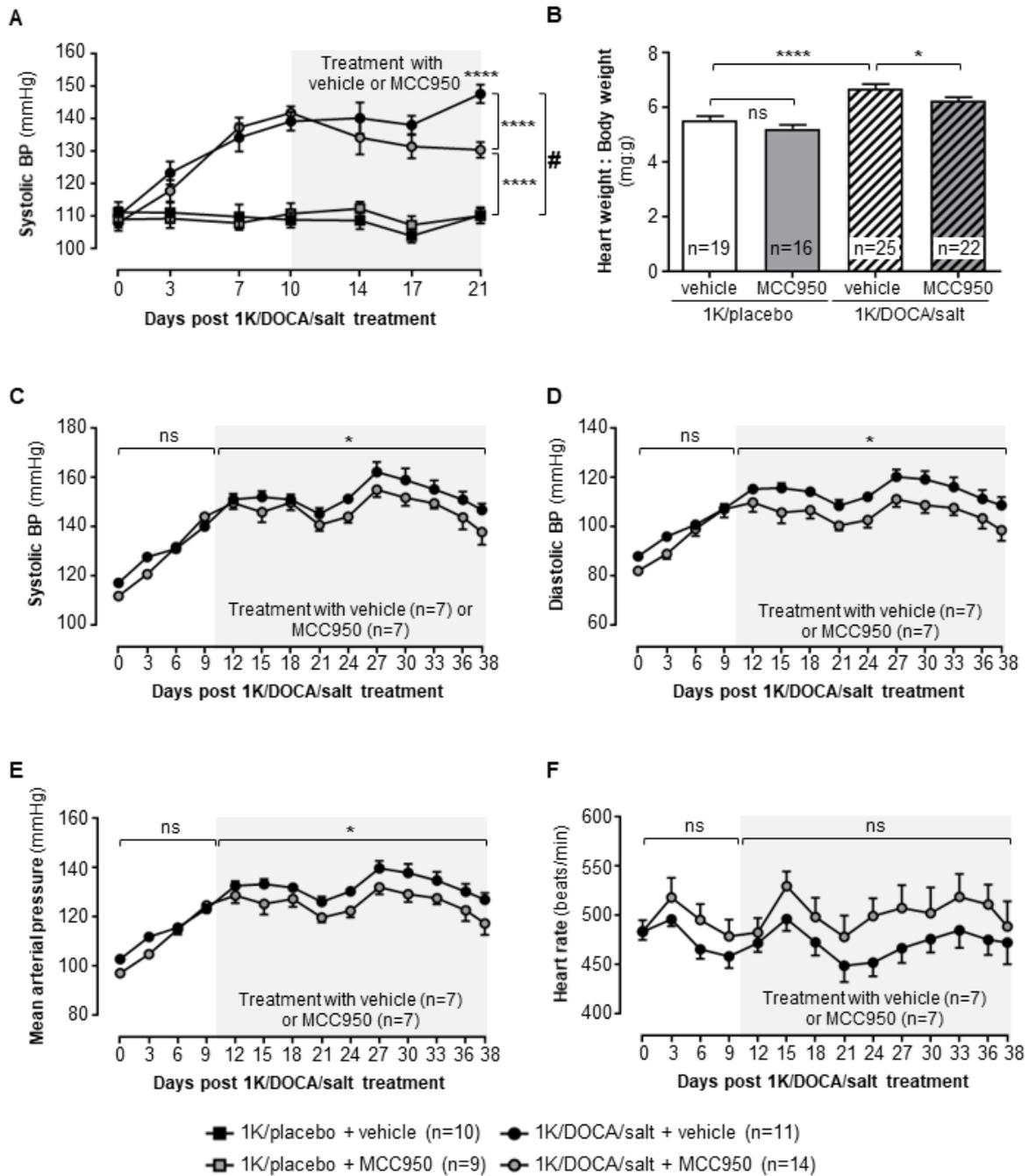
Figure 1

Figure 1: MCC950 reduces blood pressure (BP) and cardiac hypertrophy in mice with 1K/DOCA/salt-induced hypertension. Effect of MCC950 on systolic BP measured using tail-cuff plethysmography (A) and cardiac hypertrophy (B). In a separate cohort of mice, the effects of 1K/DOCA/salt and MCC950 on systolic BP (C), diastolic BP (D), mean arterial pressure (E) and heart rate (F) were measured by radiotelemetry at 10 minute-intervals and plotted as an average over a 3-day period. All values are expressed as mean ± S.E.M. *$P<0.05$, ****$P<0.0001$, ns=not significant for one-way ANOVA or two-way repeated-measures ANOVA followed by Newman-Keuls *post-hoc* test as appropriate.
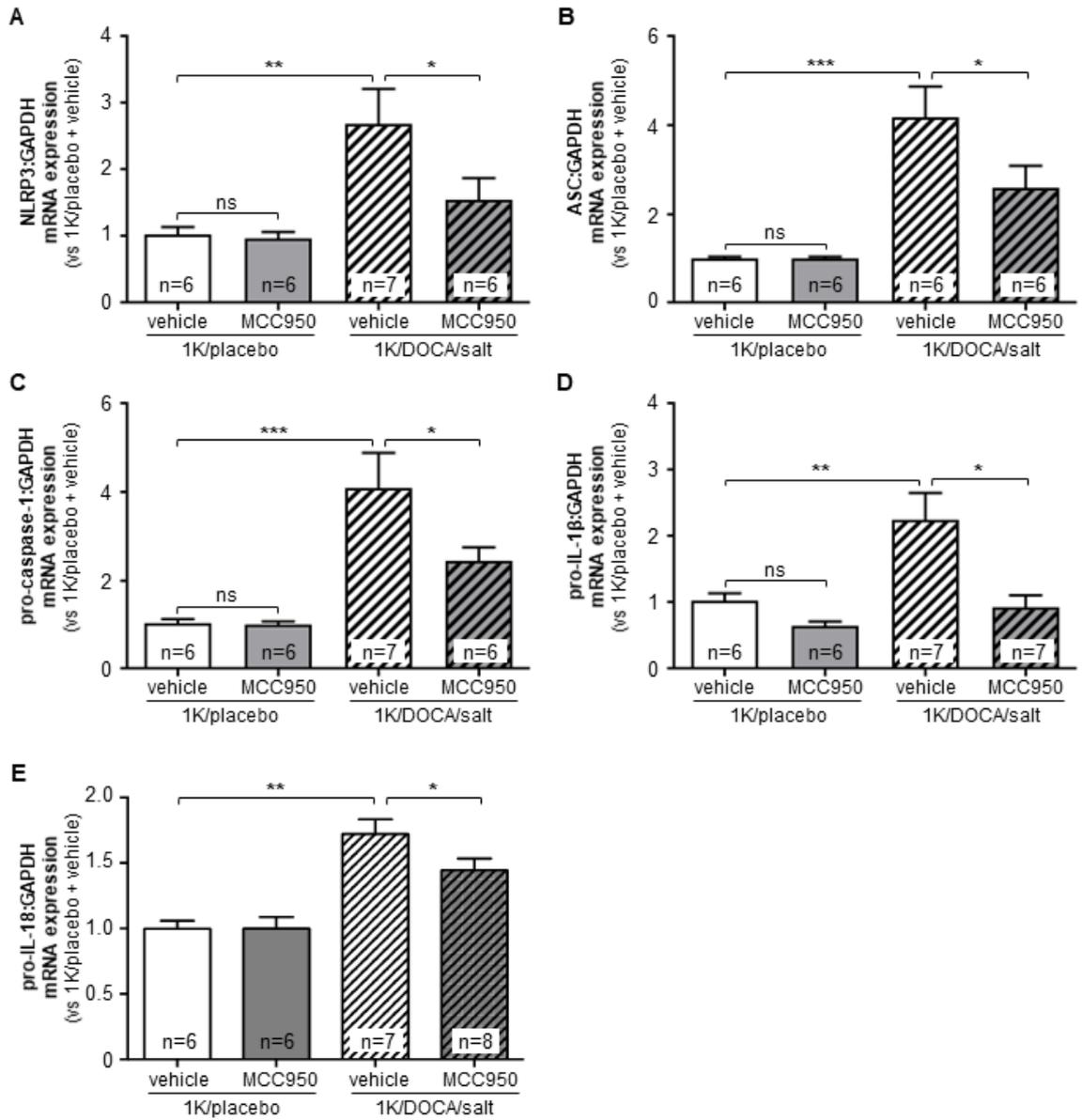
Figure 2

Figure 2: MCC950 reduces inflammasome priming in the kidneys of mice with 1K/DOCA/salt-induced hypertension. Effect of MCC950 on renal mRNA expression of NLRP3 (A), ASC (B), pro-caspase-1 (C), pro-interleukin (IL)-1β (D) and pro-IL-18 (E) in mice treated with either 1K/DOCA/salt or 1K/placebo. Messenger RNA expression was measured using the comparative Ct method against GAPDH expression. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, ns=not significant for two-way ANOVA followed by Newman-Keuls *post-hoc* test.
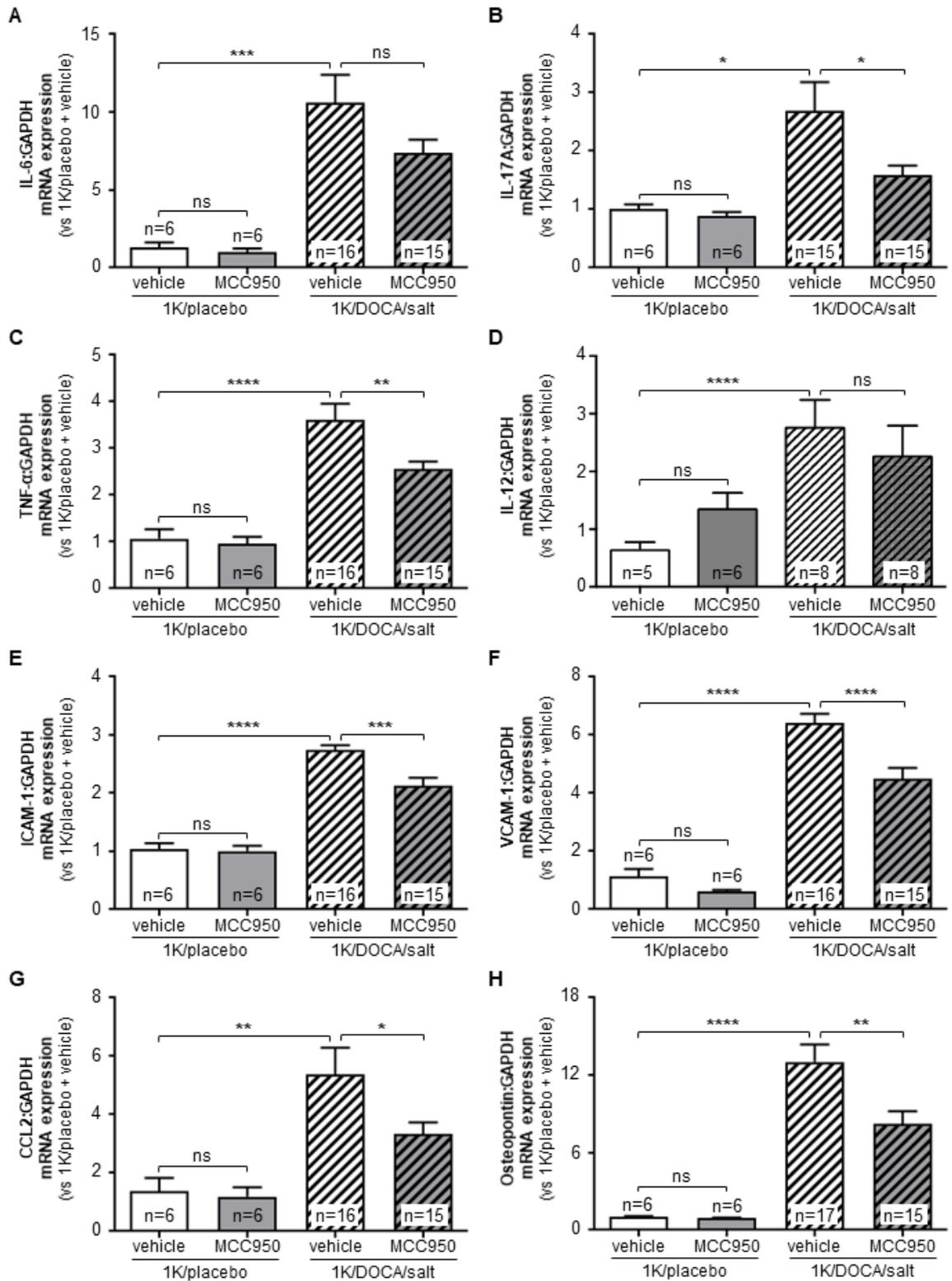
Figure 3

Figure 3: MCC950 reduces the expression of several renal inflammatory markers in mice with 1K/DOCA/salt-induced hypertension. Effect of MCC950 on renal mRNA expression of IL-6 (A), IL-17A (B), tumour necrosis factor-α (TNF-α; C), IL-12 (D), intercellular adhesion molecule-1 (ICAM-1; E), vascular cell adhesion molecule-1 (VCAM-1; F), chemokine C-C motif ligand 2 (CCL2; G) and osteopontin (H). Messenger RNA expression was measured using the comparative Ct method against GAPDH expression. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns=not significant for two-way ANOVA followed by Newman-Keuls *post-hoc* test.
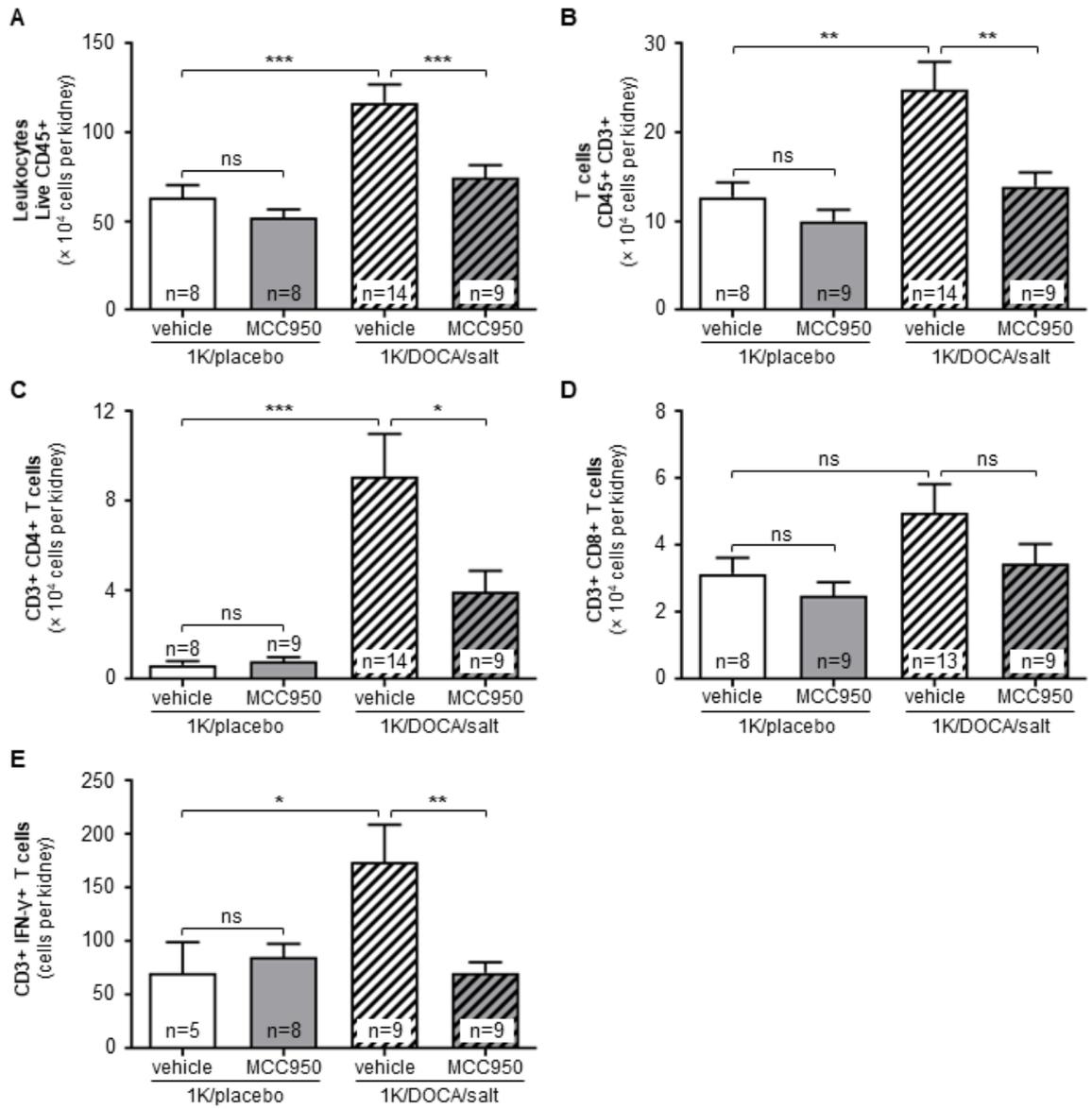
Figure 4

Figure 4: MCC950 reduces T cell accumulation in the kidneys of mice with 1K/DOCA/salt-induced hypertension. Flow cytometric analysis showing the effect of MCC950 on accumulation of total CD45$^+$ leukocytes (A), total CD3$^+$ T cells (B), CD4$^+$ T cells (C), CD8$^+$ T cells (D), and interferon-gamma$^+$ (IFN-γ^+) T cells (E) in the kidneys of mice treated with either 1K/DOCA/salt or 1K/placebo. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns=not significant for two-way ANOVA followed by Newman-Keuls *post-hoc* test.
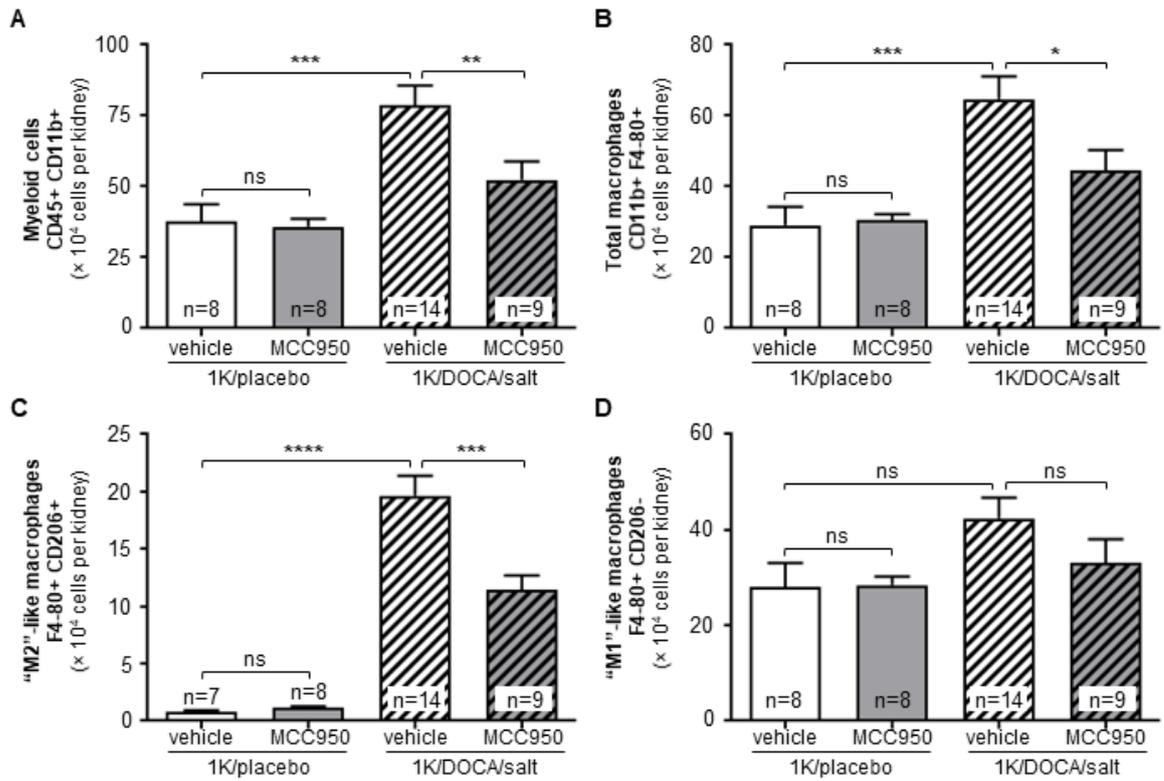
Figure 5

Figure 5: MCC950 reduces macrophage accumulation in the kidneys of mice with 1K/DOCA/salt-induced hypertension. Flow cytometric analysis showing the effect of MCC950 on accumulation of total $CD45^+CD11^+$ myeloid lineage cells (A), total $F4/80^+$ macrophages (B), $CD206^+$ M2-like macrophages (C) and CD206 M1-like macrophages (D) in the kidneys of mice treated with either 1K/DOCA/salt or 1K/placebo. Values are expressed as mean ± S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, ns=not significant for two-way ANOVA followed by Newman-Keuls *post-hoc* test.
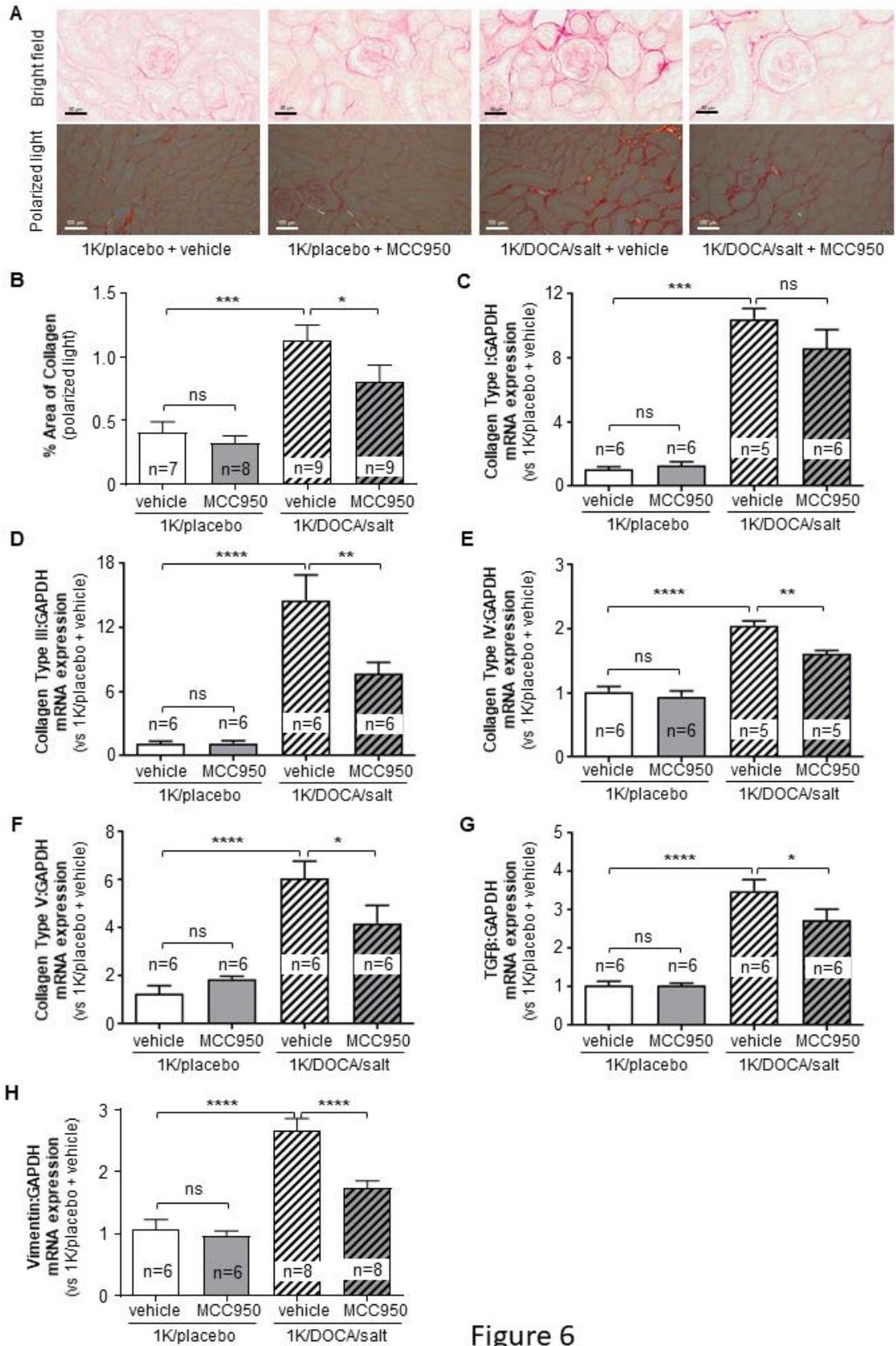
Figure 6

Figure 6: MCC950 reduces renal interstitial fibrosis in mice with 1K/DOCA/salt-induced hypertension. Effect of MCC950 on interstitial collagen deposition (A and B) and mRNA expression levels of the collagen α-subunits Type I (C), Type III (D), Type IV (E) and Type V (F), transforming growth factor-β (TGF-β; G) and vimentin (VIM; H) in kidneys of mice treated with either 1K/DOCA/salt or 1K/placebo. Representative bright-field picrosirius red stained images are shown at 40x magnification (scale = 50 µm; A). Polarised picrosirius red stained images are shown at 20x magnification (scale = 50 µm) Messenger RNA expression was measured using the comparative Ct method against GAPDH expression. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns=not significant for two-way ANOVA followed by Newman-Keuls *post-hoc* test.
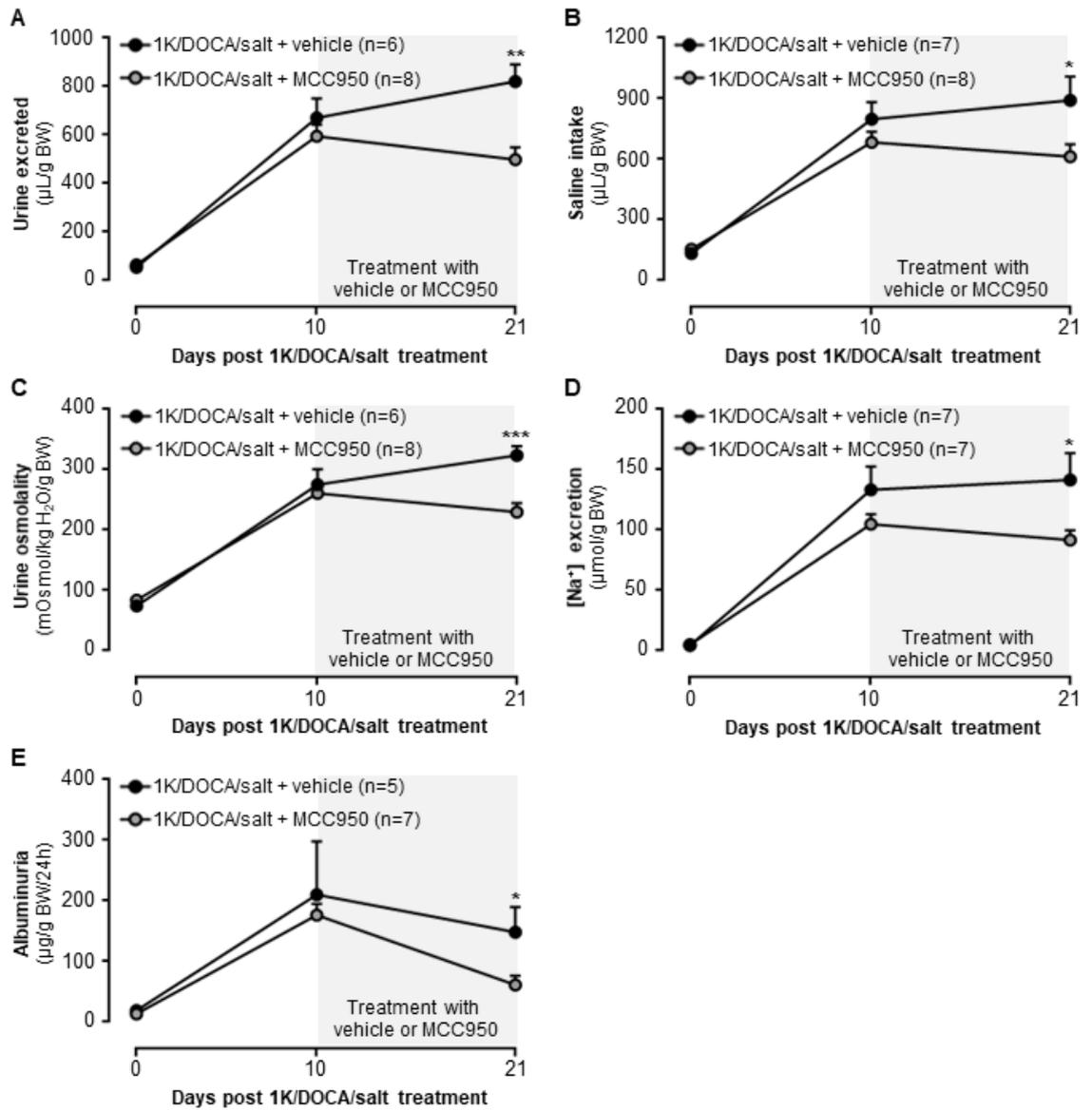
Figure 7

Figure 7: MCC950 improves kidney function in mice with 1K/DOCA/salt-induced hypertension. Effect of MCC950 on volume of urine excreted (A), volume of saline consumed (B), urine osmolality (**C**), urine [Na$^+$] (**D**) and albuminuria (**E**) in mice treated with either 1K/DOCA/salt or 1K/placebo. Values are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 for two-way repeated measures ANOVA followed by Newman-Keuls *post-hoc* test.

Chapter 7:

General Discussion

Discussion

This project examined the impact of inhibiting inflammasome-mediated inflammation on the pathogenesis of 1K/DOCA/salt-dependent hypertension in mice using a combination of pharmacological and genetic approaches. The major take home message is that irrespective of which level the inflammasome signalling system is targeted (i.e. at the level of enzyme itself or its cytokine products), there are clear benefits in terms of disease outcomes. Thus, selective inhibition of IL-1β signalling with anakinra in mice with established hypertension, reduced blood pressure and renal fibrosis, despite having little effect on the expression of renal inflammatory markers. By contrast, selective life-long deficiency of IL-18 signalling, as applies to IL-18$^{-/-}$ mice, was not only associated with profound protection against 1K/DOCA/salt-induced changes in blood pressure, but also with markedly reduced renal inflammation and fibrosis. Finally, studies utilising a small molecule inhibitor of NLRP3 inflammasome activity, MCC950, showed that blocking inflammasome-mediated signalling at the level of NLRP3 assembly was also an effective strategy for reducing blood pressure, renal inflammation, renal fibrosis, and renal dysfunction in the 1K/DOCA/salt model. In the following chapter, I will discuss the above findings collectively, focussing on where they have positioned the field in terms of clinical translation, which inhibitory strategies are likely to be of most clinical utility going forward, and which questions remain unanswered.

7.1 Contribution of the NLRP3 versus other inflammasomes isoforms to the pathogenesis of hypertension

While the use of MCC950 in Chapter 6 suggests a key role for the NLRP3 inflammasome in the pathogenesis of hypertension and renal inflammation, it must be acknowledged that other inflammasome isoforms exists and could potentially play a role in the disease process. There are two main reasons for why we initially focussed on the NLRP3 inflammasome in this study. Firstly, the NLRP3 inflammasome is the most well characterised of all the

inflammasome isoforms and has been implicated as a major contributor to inflammation in many 'classical' inflammatory diseases including rheumatoid arthritis [1], gout [2], lupus [3] and atherosclerosis [1], and even certain metabolic diseases such as Type II diabetes [4]. Indeed, it has even been suggested that a primary function of the NLRP3 inflammasome is to detect disturbances in cellular homeostasis mechanisms [1], making it a strong candidate for the mediator of inflammation in a disease such as hypertension involving massive changes to homeostatic mechanisms in numerous tissues. The second reason for focussing on NLRP3 was a more practical one. As NLRP3 has been the focus of such a large amount of previous research, there exists a wide variety of research tools allowing for its selective inhibition, one of which is the recently-developed, small molecule inhibitor, MCC950, used in the current study. However, while we have shown that the NLRP3 inflammasome is an important driver of IL-1β- and IL-18-mediated inflammation in hypertension, the maturation of these cytokines could have additionally been driven by pathways independent of the NLRP3 inflammasome. Caspase-1 is the inflammasome-associated enzyme that cleaves pro-IL-1β and pro-IL-18 into their mature and active forms. Therefore, any inflammasome complex that is coupled to caspase-1 activation has the potential to promote upregulation of IL-1β and IL-18 [5]. Other inflammasome subtypes consisting of caspase-1 include AIM2, IFI6, and NLRC4 [6]. Interestingly, both AIM2 and IFI6 also rely on the ASC subunit to couple the pattern recognition domain to caspase-1 [7,8]. Therefore, a role for one or both of these inflammasomes in the pathophysiology of hypertension might provide an explanation for why, in our previous study [7], ASC-deficiency proved so much more effective at reducing BP than did NLRP3 inhibition in the present study. The concept of multiple inflammasomes being involved in the pathophysiology of hypertension also raises the possibility that multiple cell types could be responsible for elevated inflammasome activity and IL-1β/IL-18 production. While NLRP3 appears to be the main inflammasome isoform expressed in macrophages [9], non-immune cells such as tubular epithelial cells have been shown to express the AIM2 isoform [10] in addition to the NLRP3 inflammasome [11]. Therefore, it will therefore be interesting to determine whether mice that are deficient

in AIM2, or indeed other inflammasome isoforms, are similarly afforded protection against 1K/DOCA/salt-dependent and/or other forms of hypertension.

7.2 Inhibition of IL-1β signalling as an approach to treat hypertension

Chapter 3 revealed that while there was some benefits in inhibiting IL-1β signalling in hypertension in terms of blood pressure reductions, this approach was not nearly as effective as NLRP3 or IL-18 inhibition at protecting against renal inflammation and damage. In fact, anakinra actually promoted renal hypertrophy, suggesting that it may have even caused further damage to the kidneys. Being a human protein, the introduction of anakinra into mice had the potential to evoke an immune response in its own right, which could have contributed to the kidney damage. Indeed, our findings that antibody levels were elevated in mice treated with anakinra certainly supports this possibility and suggests that mouse models of hypertension may not be the optimal way of investigating the effectiveness of anakinra as a therapy. Regardless of this limitation of the present study, it should be noted that anakinra is primarily metabolised and inactivated by the kidneys [12,13] and that because of this, it is contraindicated in patients with reduced renal function. As impaired renal function is a common comorbidity in many hypertensive patients, we would suggest that anakinra is unlikely to be of any major benefit for the future treatment of human hypertension.

Canakinumab is a more recently developed biological that is used to target IL-1β signalling [14]. It is a human monoclonal neutralising antibody that targets IL-1β without interfering with IL-1α [15]. Canakinumab has a better pharmacokinetic profile and a longer half-life than anakinra and has been approved for the treatment of several auto-inflammatory syndromes including Muckle-Wells syndrome, familial cold auto-inflammatory syndrome and neonatal-onset multisystem inflammatory disease [14]. As mentioned earlier in this thesis, canakinumab was recently tested in a Phase III clinical trials for its efficacy in several chronic illnesses including cardiovascular diseases [15]. The findings from that study were

highly encouraging and showed that a bolus dose of 150 mg of canakinumab once every three months, resulted in a 15% reduction in deaths due to heart attacks, strokes and cardiovascular diseases. The treatment was also associated with a reduction in the serum levels of C-reactive protein which is a marker of general inflammation. Surprisingly, there was no information released on whether canakinumab therapy reduced blood pressure or improved renal functions, and thus we anxiously await the release of further findings from this major study [15].

7.3 Inhibition of IL-18 signalling as an approach to treat hypertension

Chapter 4 highlighted the potential of inhibiting IL-18 signalling in the treatment of hypertension. In contrast to IL-1β inhibition with anakinra, genetic deficiency of IL-18 was highly effective at inhibiting both the elevated blood pressure response and the renal inflammation and damage in the 1K/DOCA/salt model of hypertension. Clearly, one must be cautious in trying to draw comparisons on the relative effectiveness of targeting IL-1β versus IL-18 as an approach to treat hypertension when the former was achieved by a pharmacological intervention and the latter through life-long genetic deficiency. Therefore, it will be interesting in future studies to investigate if pharmacological interventions with inhibitors of IL-18 - for example a neutralising monoclonal antibody against IL-18 [16] or a human recombinant IL-18BP [17] – proves to be as effective at reducing hypertension and renal inflammation as IL-18 gene deficiency.

A common approach for inhibiting pathophysiological signalling pathways for the treatment of diseases is to identify and target the receptor(s) that is responsible for initiating such signalling. An interesting finding from Chapter 4 was that adoptively transferring IL-18R1-deficient T cells into hypertensive RAG1$^{-/-}$ mice (a strain of mice devoid of T cells) did not protect them against the development of 1K/DOCA/salt hypertension. This suggests that targeting IL-18R1- the main subunit of the IL-18 receptor complex, may not be a valid approach for inhibiting IL-18 signalling. As mentioned earlier in this thesis, IL-37 is a recently

identified member of the IL-1 family, which in contrast to IL-1β and IL-18, has been shown to have anti-inflammatory properties [18,19]. Although a mouse homologue has not yet been identified, in humans, IL-37 is known to compete with IL-18 for the IL-18R1 and, upon binding, recruits an entirely different set of accessory proteins, namely IL-1R8 and IL-1R9 as opposed to IL-18RAP. The recruitment of IL-1R8 and IL-1R9 is thought to skew IL-18R1 signalling along an anti-inflammatory pathway [19,20]. Therefore, targeting the accessory protein, namely IL-18RAP, that is specifically responsible for coupling the IL-18R1 to a pro-inflammatory signalling cascade may be more effective than targeting IL-18R1 itself, which could block both pro- and anti-inflammatory signalling.

7.4 Nitric oxide (NO) donors as novel inhibitors of inflammasome signalling

Chapter 5 highlighted NO donors as powerful inhibitors of NLRP3 inflammasome priming and IL-1β release, at least in an *in vitro* setting. Obviously, further investigations are required to fully characterise the molecular mechanisms of NO donor-mediated inhibition of inflammasome priming. Nevertheless, it would be interesting to conduct *in vivo* studies to determine if NO donors are not only effective at reducing blood pressure in experimental hypertension models, but also the associated end-organ inflammation, and whether these effects are the result of inhibition of inflammasome activity. Furthermore, as NO donors (such as glyceryl trinitrate (GTN)) are currently used to treat angina in the clinic [21–23], it would be interesting to determine whether these patients display reduced inflammasome activity i.e. reduced circulating IL-1β or IL-18 levels, compared to untreated patients. It is also interesting to note that treatment with traditional NO donors such as GTN are prone to the development of tolerance which is the result of a depletion in the co-factors required to bio-convert the drug to NO. Hence, this further highlights the possible use of NO donors that do not require this bio-conversion step (such as SPER-NO) in the treatment of end-organ inflammation and high blood pressure associated with hypertension.

7.5 Risk versus benefit of immune-modulating therapies for the treatment of hypertension

An important consideration in light of the previous discussion is whether the benefits of inhibiting inflammasome/IL-1β/IL-18 signalling for the treatment of hypertension are likely to outweigh the risks associated with immunosuppression. Indeed, while the CANTOS trial demonstrated the cardiovascular benefits of IL-1β suppression with canakinumab, it also highlighted an increased incidence of fatal infections resulting from a reduction in neutrophil counts leading to immunosuppression [15]. Thus, in patients with (a) low-grade or moderate hypertension, (b) a limited number of risk factors for cardiovascular disease (other than hypertension), and/or (c) whose blood pressure is well controlled by conventional therapies such as ACE inhibitors, β-adrenergic receptor antagonists, Ang II Type 1 receptor antagonists, and/or diuretics, it is hard to envisage the benefits of inflammasome/IL-1β/IL-18 signalling outweighing the risks of immunosuppression. By contrast, in patients with severe and/or resistant hypertension (i.e. blood pressure not controlled despite taking 3 or more conventional medications [24]), or who have multiple risk factors for cardiovascular disease, it is conceivable that a benefit-risk analysis may support the use of immune modulating therapies. Finally, it may be possible to mitigate the risk of infection with inflammasome/IL-1β/IL-18-inhibiting therapies (and sway the benefit-risk equation even further to the left) through short-term or intermittent dosing regimens that aim to get inflammation component of the condition under control, so that conventional therapies can then be used to keep the condition in check.

Conclusions

Hypertension has traditionally been considered a disease involving dysregulation of three key physiological systems, namely the sympathetic nervous system, the kidneys and the vasculature. Indeed, this is reflected by current treatment strategies which revolve around the use of various combinations of diuretics, vasodilators, and beta-blockers. This thesis

provides compelling evidence that the immune system and in particular the inflammasome should also be considered alongside the above systems as an important pathogenic mechanism in hypertension and a potential target for future therapies.

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Appendix

Themed Section: Inflammation: maladies, models, mechanisms and molecules

RESEARCH PAPER

Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice

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BACKGROUND AND PURPOSE

Inflammasomes are multimeric complexes that facilitate caspase-1-mediated processing of the pro-inflammatory cytokines IL-1β and IL-18. Clinical hypertension is associated with renal inflammation and elevated circulating levels of IL-1β and IL-18. Therefore, we investigated whether hypertension in mice is associated with increased expression and/or activation of the inflammasome in the kidney, and if inhibition of inflammasome activity reduces BP, markers of renal inflammation and fibrosis.

EXPERIMENTAL APPROACH

Wild-type and inflammasome-deficient ASC$^{-/-}$ mice were uninephrectomized and received deoxycorticosterone acetate and saline to drink (1K/DOCA/salt). Control mice were uninephrectomized but received a placebo pellet and water. BP was measured by tail cuff; renal expression of inflammasome subunits and inflammatory markers was measured by real-time PCR and immunoblotting; macrophage and collagen accumulation was assessed by immunohistochemistry.

KEY RESULTS

1K/DOCA/salt-induced hypertension in mice was associated with increased renal mRNA expression of inflammasome subunits NLRP3, ASC and pro-caspase-1, and the cytokine, pro-IL-1β, as well as protein levels of active caspase-1 and mature IL-1β. Following treatment with 1K/DOCA/salt, ASC$^{-/-}$ mice displayed blunted pressor responses and were also protected from increases in renal expression of IL-6, IL-17A, CCL2, ICAM-1 and VCAM-1, and accumulation of macrophages and collagen. Finally, treatment with a novel inflammasome inhibitor, MCC950, reversed hypertension in 1K/DOCA/salt-treated mice.

237

CONCLUSIONS AND IMPLICATIONS

Renal inflammation, fibrosis and elevated BP induced by 1K/DOCA/salt treatment are dependent on inflammasome activity, highlighting the inflammasome/IL-1β pathway as a potential therapeutic target in hypertension.

LINKED ARTICLES

This article is part of a themed section on Inflammation: maladies, models, mechanisms and molecules. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2016.173.issue-4

Abbreviations

AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a carboxy-terminal CARD; CARD, caspase activation and recruitment domain; CCL, C-C motif chemokine ligand; DAMP, danger-associated molecular pattern; DOCA, deoxycorticosterone acetate; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule-1; NLRC4 (IPAF), NOD-like receptor family CARD domain-containing protein 4; NLRP, NOD-like receptor family pyrin domain-containing protein; PAMP, pathogen-associated molecular pattern; VCAM-1, vascular cell adhesion molecule-1

Tables of Links

TARGETS	LIGANDS			
Catalytic receptors[a]	Allopurinol	CCL2	IFN-γ	LPS
NLRC4 (IPAF)	Anakinra	CCL5	IL-1β	MCC950
NLRP1	Angiotensin II	Clexane	IL-6	Osteopontin
NLRP3	ATP	Deoxycorticosterone	IL-17A	Rilonacept
Enzymes[b]	Biotin	ICAM-1	IL-18	TNF
Caspase 1	Canakinumab		IL-23	VCAM-1

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander et al., 2013a,b).

Introduction

Hypertension is now recognized as a chronic, low-grade inflammatory disease, with the kidneys representing a major site of this inflammation (Harrison et al., 2011; Rodriguez-Iturbe et al., 2012). Through tight regulation of the pressure–natriuresis relationship, whereby increments in BP are accompanied by a compensatory reduction in sodium reabsorption in the proximal tubules, the kidneys play a major role in BP homeostasis (Rodriguez-Iturbe et al., 2012). By promoting such processes as the intrarenal accumulation of T-cells and macrophages (Mattson, 2014; Wei et al., 2014), a loss of the peritubular vascular network (Rodriguez-Iturbe and Johnson, 2010), up-regulation of Na⁺ transport/re-uptake mechanisms (Meng et al., 2006; Aoi et al., 2007), and interstitial collagen production (Mezzano et al., 2001; Blasi et al., 2003), inflammation may blunt the pressure–natriuresis relationship and thereby contribute to hypertension. Hence, a better understanding of the processes that give rise to renal inflammation, may pave the way for novel anti-hypertensive therapies.

Inflammasomes are sensors of the innate immune system that play important roles in initiating inflammation in response to acute infections and chronic diseases. Inflam-

masomes are multimeric complexes that can be activated by a diverse range of pathogen- and danger-associated molecular patterns ('PAMPs' and 'DAMPs', respectively), including bacterial pore-forming toxins such as nigericin (Mariathasan et al., 2006), crystalline substances such as uric acid (Martinon et al., 2006) and cholesterol (Duewell et al., 2010), misfolded and/or acute phase proteins such as β-amyloid (Halle et al., 2008) and serum amyloid A (Niemi et al., 2011), and extracellular ATP (Mariathasan et al., 2006). To date, four inflammasome complexes have been described, each displaying selectivity towards a different suite of PAMPs and DAMPs. Inflammasomes are comprised of one of four distinct pattern recognition receptors – NOD-like receptor family pyrin domain-containing protein (NLRP) 1, NLRP3, ICE protease-activating factor/NOD-like receptor family CARD domain-containing protein 4 (NLRC4) or absent in melanoma 2 (AIM2) – and it is this sensor that defines the inflammasome and confers its selectivity (Latz, 2010; Schroder and Tschopp, 2010a). Once activated, the inflammasome recruits pro-caspase-1, either via an adapter protein, ASC [apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)], or homotypically using its own CARD. The clustering of several pro-caspase-1 subunits at the inflammasome complex results in auto-cleavage and

formation of active caspase-1 heterodimers, each consisting of a p10 and p20 subunit. These active caspase-1 heterodimers convert inactive cytosol-bound pro-IL-1β and pro-IL-18 into their mature forms, IL-1β and IL-18, respectively, which can be released from the cell to activate pro-inflammatory signalling pathways in both an autocrine and paracrine manner (Latz, 2010; Schroder and Tschopp, 2010a).

As a safeguard against unwanted inflammatory responses, expression of inflammasome components and of the cytokine precursors, pro-IL-1β and pro-IL-18, are normally kept at very low levels within cells (Dinarello, 1996; 1999; Bauernfeind *et al.*, 2009). This requires the system to be 'primed' before inflammasome activation and IL-1β/IL-18 production can commence. Inflammasome priming normally occurs downstream of the transcription factor NF-κB (Bauernfeind *et al.*, 2009) and essentially involves trans-activation of the various genes that encode the different inflammasome subunits and their cytokine targets. Stimuli known to lead to inflammasome priming include toll-like receptor ligands such as LPS, cytokines such as TNF and reactive oxygen species (Kahlenberg *et al.*, 2005; Bauernfeind *et al.*, 2009; 2011).

The NLRP3 inflammasome is the most widely studied of the four inflammasomes identified. Unlike other inflammasomes which are activated by a limited array of specific PAMPs or DAMPs, such as the anthrax lethal toxin and muramyl dipeptide (NLRP1), cytosolic flagellin of various bacteria (NLRC4), or cytosolic double-stranded DNA (AIM2), the NLRP3 inflammasome can be activated by multiple stimuli including LPS, reactive oxygen species, ATP and microcrystals, and is thus believed to be the most important isoform contributing to inflammation in the setting of chronic diseases (Schroder and Tschopp, 2010a; Schroder *et al.*, 2010b; Wen *et al.*, 2012; Ozaki *et al.*, 2015).

Previous studies have shown that hypertension in humans is associated with increased circulating levels of IL-1β and IL-18 (Dalekos *et al.*, 1997; Rabkin, 2009; Krishnan *et al.*, 2014; Barbaro *et al.*, 2015). Hence, in the present study, we investigated whether hypertension induced by uninephrectomy and subsequent treatment with deoxycorticosterone acetate and salt in mice (1K/DOCA/salt) is associated with an increase in inflammasome expression and or activity in the kidneys. Furthermore, we evaluated whether inhibition of inflammasome activity, either due to a genetic deficiency in the adaptor protein, ASC, or as a result of treatment with a novel NLRP3 inhibitor, was effective at reducing renal inflammation, fibrosis and hypertension in response to 1K/DOCA/salt treatment.

Methods

Animals

Male wild-type and ASC$^{-/-}$ mice (Mariathasan *et al.*, 2004), fully backcrossed onto a C57BL6/J background, aged 10–12 weeks, and weighing 25–30 g were used in this study. Mice were obtained from Monash Animal Services (Clayton, Victoria, Australia) and housed in standard mouse boxes with *ad libitum* access to normal chow and water. All procedures on mice were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition) and were approved by the Monash University

Animal Research Platform Animal Ethics Committee (Ethics number: MARP/2013/043). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

One kidney/deoxycorticosterone acetate/salt model of hypertension

All surgeries were performed under anaesthesia induced by inhalation of 2% isoflurane. While under anaesthesia, hind-paw withdrawal, blink reflexes and respiratory rate were monitored. Hypertension was induced in wild-type and ASC$^{-/-}$ mice by removal of their left kidney, implantation of a deoxycorticosterone acetate pellet (DOCA; 2.4 mg·day^{-1}, s.c.; Innovative Research of America, Sarasota, FL, USA) and replacement of their drinking water with 0.9% saline (1K/DOCA/salt) (Manhiani *et al.*, 2009). Normotensive controls for these experiments were mice that were also uninephrectomized but received a placebo pellet (Innovative Research of America) and normal drinking water (1K/placebo).

Angiotensin II-infusion model of hypertension

In another cohort of wild-type and ASC$^{-/-}$ mice, hypertension was instead induced by implantation of a micro-osmotic pump (Model 1004, Alzet, Cupertino, CA, USA) containing angiotensin II (to deliver 0.7 mg·kg^{-1}·day^{-1}, s.c.; Moore *et al.*, 2013). Normotensive controls received the vehicle for angiotensin II (i.e. 0.9% saline; 0.11 μL·h^{-1}, s.c.) via micro-osmotic pumps.

Administration of MCC950 to mice with 1K/DOCA/salt-induced hypertension

A separate cohort of mice were treated with MCC950; a novel diarylsulfonylurea-based compound that acts as a selective inhibitor of NLRP3 inflammasome activity by preventing oligomerization of the complex (Coll *et al.*, 2015). MCC950 was administered as an intervention in mice with established hypertension. Briefly, hypertension was induced in mice by 1K/DOCA/salt treatment as described above. After 10 days (i.e. when BP had increased to a stable plateau), a micro-osmotic pump was implanted to release either MCC950 (10 mg·kg^{-1}·day^{-1}, s.c.) or vehicle (saline; 0.5 μL·h^{-1}, s.c.) for the remaining 11 days of treatment.

BP measurements

Systolic BP was measured by tail cuff plethysmography (MC4000 Multi Channel Blood Pressure Analysis System, Hatteras Instruments, Cary, NC, USA; Krege *et al.*, 1995). BP was recorded everyday for 3 days prior to surgery in order to acclimatize mice to the procedure. BP was then measured just prior to surgery (day 0) and then re-measured after 3, 7, 10, 14, 21 and 28 days.

Gene expression in the kidney

At the end of the treatment period, mice were killed by isoflurane overdose and perfused through the left ventricle with PBS containing 0.2% 400 IU of Clexane (Sanofi Aventis, Paris, France). The right kidney was excised, cut in half transversally, snap-frozen in liquid nitrogen, and stored at −80°C until time of assay. RNA was extracted from one quarter of the

kidney using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The yield and purity of RNA was determined using a Nano-Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was converted to cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. The cDNA was then used as a template in real-time PCR to measure mRNA expression of NLRP3, ASC, pro-caspase-1, pro-IL-1β, pro-IL-18, IL-6, IL-17A, IFN-γ, TNF, C-C motif chemokine ligand (CCL) 2, CCL5, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), or the house keeping gene, GAPDH (TaqMan Gene Expression Assays, Applied Biosystems). Real-time PCR was performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and the comparative C_t method was used to calculate the fold-change in mRNA expression relative to a reference control sample (Schmittgen and Livak, 2008).

Caspase-1 and IL-1β protein expression in the kidney

Protein lysates were prepared from the other quarter of the kidney in 1.5× Laemmli buffer (7.5% glycerol, 3.75% β-mercaptoethanol, 2.25% SDS, 75 mM Tris-HCl pH 6.8, 0.003% bromophenol blue). One hundred and fifty µg of protein was heated at 80°C and loaded onto a 12.5% polyacrylamide gel, in parallel with a molecular weight marker (Precision Plus Protein Dual Colour Standards; Bio-Rad Laboratories). Proteins were separated according to molecular weight by electrophoresis (60 V for 30 min followed by 110 V for 2 h) and then transferred onto a 0.45 µm pore Immobilon-P PVDF membrane (Millipore Corporation, Billercia, MA, USA) using a semi-dry electroblotting transfer apparatus (25 V for 1 h; Trans-Blot Semi-Dry Transfer Cell, Bio-Rad Laboratories). The membrane was incubated overnight at 4°C with an anti-caspase-1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1:1000 or with an anti-IL-1β antibody conjugated to biotin (R&D Systems, Minneapolis, MN, USA) at a dilution of 1:250. Secondary antibodies used for detection were a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP; 1:10 000; Dako, Glostrup, Denmark) and a streptavidin antibody conjugated to Alexa Fluor 680 (1:2000; Life Technologies) respectively. To control for protein loading, the membrane was probed with a mouse anti-GAPDH antibody (1:20 000; Abcam, Cambridge, UK), and then with an HRP-conjugated goat anti-mouse secondary antibody (1:10 000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Membranes to detect the caspase-1 p45 and p10 bands were developed using a CP-1000 X-ray film processor (Agfa Corporation, Mortsel, Belgium), and quantified using a densitometer (ChemiDoc XRS Universal Hood II, Bio-Rad Laboratories) and the accompanying 1-D analysis software (Quantity One 4.6.1, Bio-Rad Laboratories), whereas the Odyssey Scanner ODY-1882 (LI-COR, Lincoln, NE, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the density of IL-1β p31 and p17 bands.

Immunohistochemistry for detection of macrophage accumulation in the kidneys

Briefly, the kidney was cut in half transversely, fixed in 10% formalin for 3 days and then embedded in paraffin. Kidneys were sectioned (5 µm), deparaffinized, rehydrated and stained with rat anti-F4/80 (Bio-Rad Laboratories), followed by a biotinylated anti-rat IgG secondary antibody (Vector Labs, Burlingame, CA, USA). An avidin-biotin complex (ABC Elite, Vector Labs) was used for signal amplification and 3,3'-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) substrate was used for detection. Finally, sections were counterstained with hematoxylin and mounted in DePex (VWR International, Radnor, PA, USA). Imaging and analysis was performed in a blinded manner. Six randomly selected fields (viewed under a magnification of ×15) were imaged per slide and fields containing blood vessels or areas with tears in the tissue were excluded. The number of cells positive for F4/80 was counted per field of view.

Picrosirius red staining for detection of renal interstitial collagen

Kidneys were fixed, sectioned and imaged (viewed under a magnification of ×20) as described above. Interstitial collagen content of the renal cortex was quantified using 0.1% Picrosirius red staining solution. Collagen staining was quantified as a percentage of the total area per field of view using ImageJ software (National Institutes of Health).

Statistical analysis

Data are expressed as mean ± SEM. Systolic BP data was analysed by two-way repeated measures ANOVA followed by Bonferroni post hoc test. Other data were analysed using either Student's unpaired t-test or one-way ANOVA followed by Bonferroni's 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 (La Jolla, CA, USA).

Results

1K/DOCA/salt-induced hypertension is associated with increased inflammasome activity in the kidney

1K/DOCA/salt treatment in C57BL/6J mice increased systolic BP by ~30 mmHg within 7–10 days, and this increase was sustained throughout the remainder of the 21 day treatment period (Figure 1A). By contrast, systolic BP remained largely unchanged over the 21 days in 1K/placebo-treated mice (Figure 1A).

TaqMan real-time PCR analysis revealed that there was a significant increase in mRNA expression of inflammasome-related genes in the kidney of 1K/DOCA/salt-induced hypertensive mice compared with 1K/placebo-treated normotensive mice. These genes included NLRP3 (~2.5-fold; Figure 1B), ASC (~3-fold; Figure 1C), pro-caspase-1 (~2.5-fold; Figure 1D) and pro-IL-1β (~4-fold; Figure 1E). There was no significant change in mRNA expression of pro-IL-18 (Figure 1F).

Western blotting with an anti-caspase-1 antibody that detects both full-length and the cleaved p10 proteolytically active caspase-1 revealed that under control conditions (i.e. 1K/placebo), wild-type mice expressed both a 45 kDa protein

240

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S M Krishnan et al.
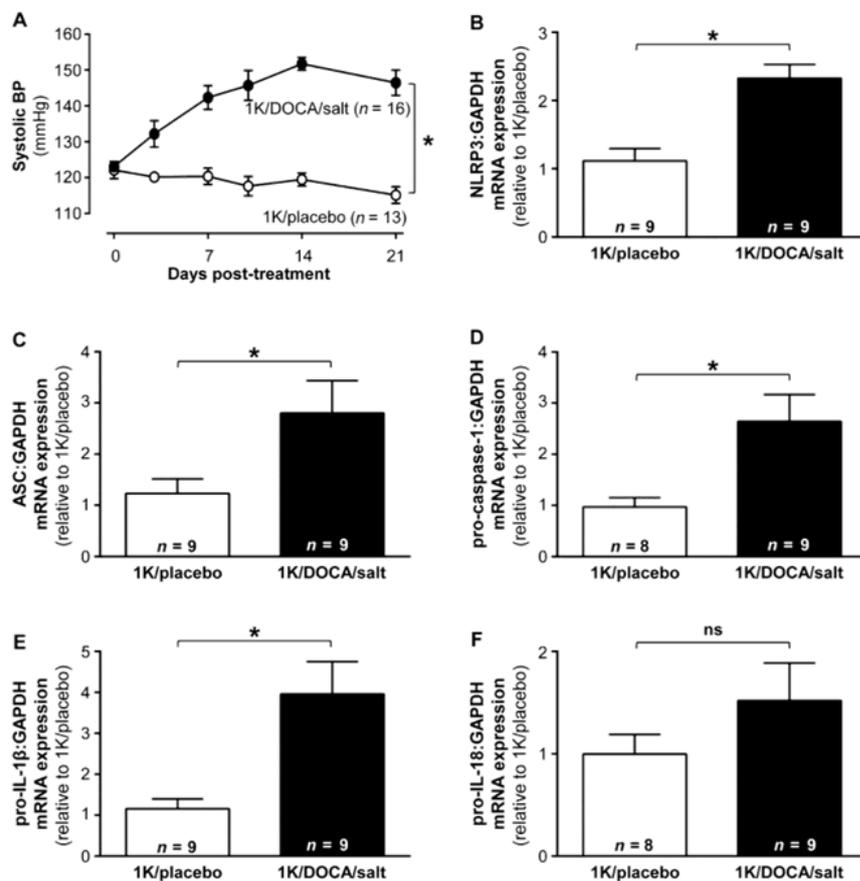
Figure 1

1K/DOCA/salt-induced hypertension in mice is associated with inflammasome priming. Effects of 1 kidney (1K)/DOCA/salt treatment in mice on systolic BP (A); and renal mRNA expression of the inflammasome-related genes NLR family, pyrin domain containing 3 (NLRP3; B); apoptosis-associated speck-like protein containing a CARD (ASC; C); pro-caspase-1 (D), pro-IL-1β (E) and pro-IL-18 (F). All values are expressed as mean ± SEM. *$P < 0.05$, ns, not significant for two-way repeated-measures ANOVA (A) or Student's unpaired t-test (B–F).

and a ~10 kDa protein that were absent in kidney samples from caspase-1^{−/−} mice and thus likely to represent the pro- and active forms of the enzyme (Figure 2A). 1K/DOCA/salt treatment was associated with an increase in renal protein expression of the pro-caspase-1 p45 subunit (i.e. by ~5-fold; Figure 2B). Levels of the active caspase-1 p10 subunit were also elevated in 1K/DOCA/salt mice (~1.5-fold; Figure 2C), albeit to a lesser extent than the p45 subunit. Thus, there was an overall reduction in the caspase-1 p10/45 ratio (~4-fold; Figure 2D). Levels of the pro-IL-1β p31 subunit (~1.5 fold; Figure 2E), active IL-1β p17 subunit (~2-fold; Figure 2F) and the ratio of IL-1β p17/p31 (~1.5-fold; Figure 2G) were all elevated in the kidneys of 1K/DOCA/salt-treated mice compared with 1K/placebo-treated mice indicative not only of increased IL-1β expression but also enhanced IL-1β processing. It is important to note, however, that despite detecting IL-1β maturation in the kidneys, IL-1β could not be detected in the plasma of 1K/DOCA/salt- or 1K/placebo-treated mice by cytometric bead array (data not shown).

ASC^{−/−} mice are protected from 1K/DOCA/salt- and angiotensin II-induced increases in systolic BP

In a separate cohort of animals, 1K/DOCA/salt treatment increased systolic BP in wild-type mice by ~40 mmHg (Figure 3A). In ASC^{−/−} mice, the pressor effect of 1K/DOCA/salt treatment was significantly blunted by approximately 40% compared with wild-type mice (Figure 3A). Furthermore, ASC^{−/−} mice were also resistant to the chronic pressor effects of another hypertensive stimulus, angiotensin II (Figure 3B), suggesting that the crucial role of the inflammasome is not only restricted to hypertension induced by 1K/DOCA/salt.

Effect of ASC deficiency on 1K/DOCA/salt-induced priming of the inflammasome

In this series of experiments, 1K/DOCA/salt treatment was again found to increase renal expression levels of the inflammasome subunit NLRP3 in wild-type mice by approximately
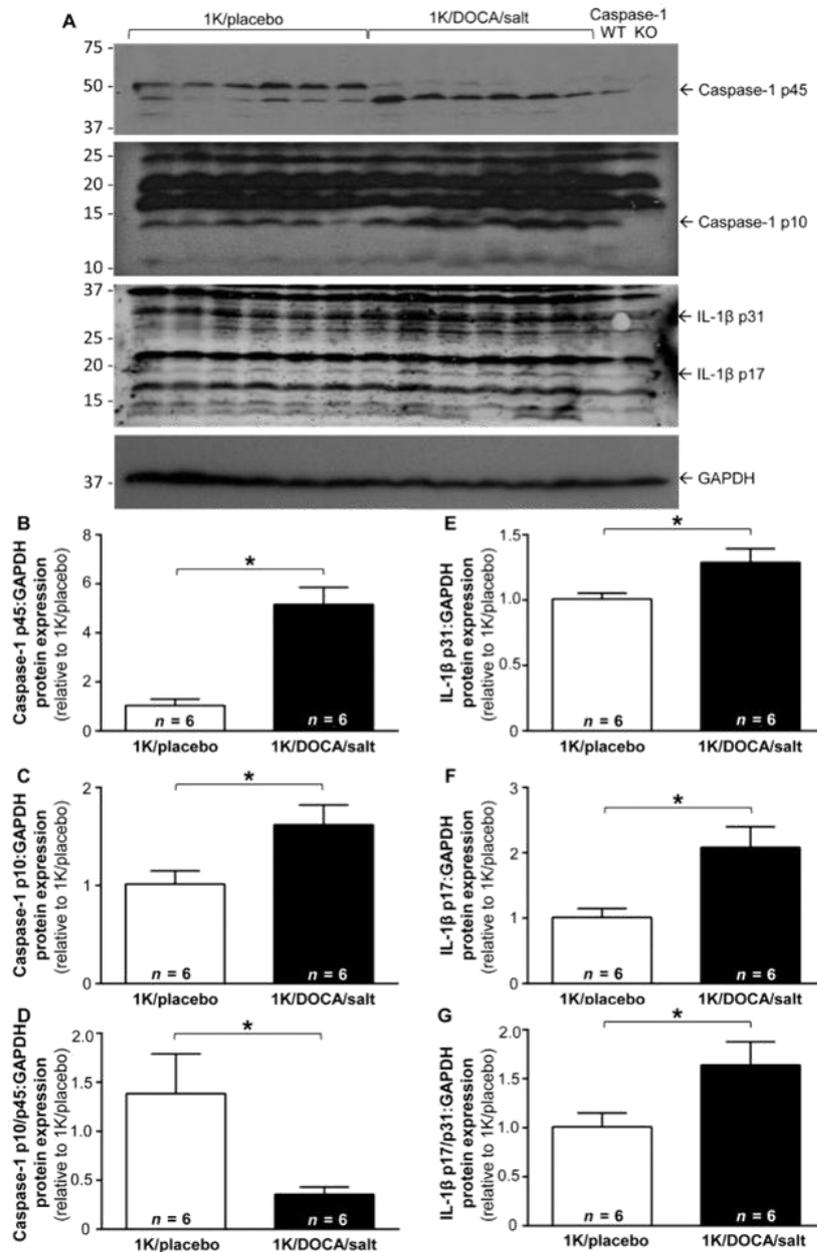
Figure 2

Effect of 1K/DOCA/salt treatment on caspase-1 and IL-1β protein expression in the kidney. A shows original Western blots in which membranes were probed with antibodies against either caspase-1 (two upper blots), IL-1β (third blot) or the housekeeping gene, GAPDH (lower blot). Note, lanes 1–6 were loaded with protein samples from normotensive (1K/placebo) mice; lanes 7–12 were samples from hypertensive [1 kidney (1K)/DOCA/salt] mice; while lanes 11 and 12 were protein samples from kidneys from littermate caspase-1^{+/+} and caspase-1^{−/−} mice, respectively, the latter serving as a negative control. Also shown are the quantified densitometric data for the uncleaved pro-caspase-1 p45 subunit (B); the active caspase-1 p10 subunit (C); the uncleaved pro-IL-1β p31 subunit (E); and the mature IL-1β p17 subunit (F), all normalized to GAPDH. (D) and (G) show the ratios of caspase-1 p10:p45 and IL-1β p17:p31 respectively. All values are expressed as mean ± SEM. *$P < 0.05$, Student's unpaired t-test.

2.5-fold (Figure 4A). Expression of NLRP3 was also elevated (compared to the normotensive controls) in ASC^{−/−} mice that were treated with 1K/DOCA/salt, although to a lesser extent (i.e. by ~1.7-fold) than that in wild-type mice (Figure 4A).

Expression of pro-caspase-1 (Figure 4B) and pro-IL-1β (Figure 4C) were increased to a similar extent in kidneys from both genotypes by treatment with 1K/DOCA/salt (Figure 4B and C). ASC expression was also up-regulated in kidneys from

242

S M Krishnan et al.
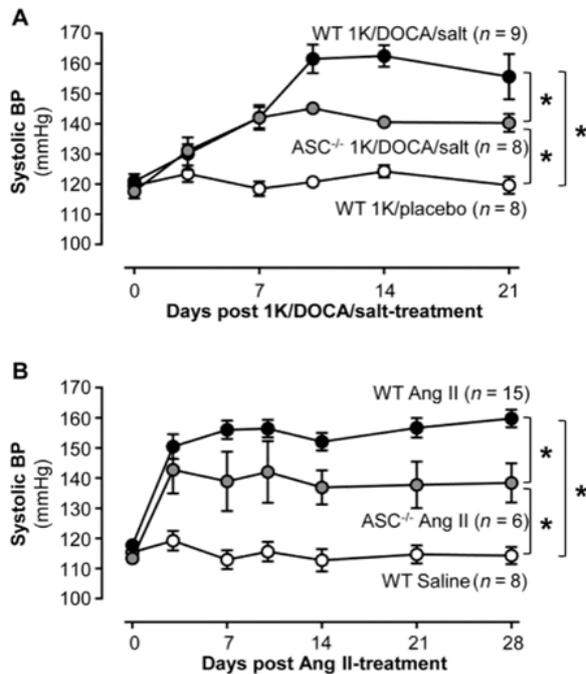
Figure 3

ASC gene deficiency protects mice from the chronic pressor effects of 1K/DOCA/salt and angiotensin II. Effects of 1 kidney (1K)/DOCA/salt (A) and angiotensin (Ang) II (B) on systolic BP in wild-type and ASC$^{-/-}$ mice. Values are expressed as mean ± SEM. *$P < 0.05$ for two-way repeated measures ANOVA.

1K/DOCA/salt-treated wild-type mice, but was barely detectable at the mRNA level in 1K/DOCA/salt-treated ASC$^{-/-}$ mice (Figure 4D), confirming that these transgenic animals were truly ASC-deficient. Again, 1K/DOCA/salt treatment appeared to have no effect on renal expression of pro-IL-18 (Figure 4E). Taken together, these results suggest that the absence of ASC only has a minor effect on inflammasome priming in the kidney during hypertension.

ASC-deficiency inhibits 1K/DOCA/salt-induced increases in expression of pro-inflammatory cytokines and macrophage infiltration of the kidney

Mature IL-1β can induce the production of the pro-inflammatory cytokines IL-6 and IL-17A (Cahill and Rogers, 2008; Sutton *et al.*, 2009; Mills *et al.*, 2013). Indeed, we showed that 1K/DOCA/salt-induced hypertension in wild-type mice was associated with significant increases in renal mRNA expression of both IL-6 and IL-17A (Figure 5A and B). In 1K/DOCA/salt-treated ASC$^{-/-}$ mice, expression of IL-6 and IL-17A was elevated relative to normotensive controls, but was nonetheless 40–60% lower than the levels observed in 1K/DOCA/salt-treated wild-type mice (Figure 5A and B). In contrast to the above, expression of IFN-γ, which lies downstream of mature IL-18, remained unchanged by 1K/DOCA/salt treatment in both wild-type and ASC$^{-/-}$ mice (Figure 5C).

Renal expression of several additional markers of inflammation including the chemokines, CCL2 (Figure 5E) and

CCL5 (Figure 5F), and the adhesion molecules, ICAM-1 (Figure 5G) and VCAM-1 (Figure 5H) were also up-regulated in 1K/DOCA/salt- versus 1K/placebo-treated wild-type mice. Expression levels of all of these genes, except CCL5, were lower in ASC$^{-/-}$ mice than in wild-type mice following 1K/DOCA/salt treatment (Figure 5). The pro-inflammatory cytokine, TNF, also appeared to be upregulated in wild-type but not in ASC$^{-/-}$ mice following 1K/DOCA/salt treatment; however, these differences failed to reach statistical significance (Figure 5D).

1K/DOCA/salt treatment in wild-type mice was also associated with a threefold increase in macrophage numbers in the kidney relative to 1K/placebo-treated mice (Figure 6B). Similar to its effects on the various markers of renal inflammation, ASC deficiency attenuated this increase by ~30% (Figure 6B). Collectively, these findings indicate that inflammasome activity is a major contributor to the renal inflammation that occurs during hypertension.

ASC$^{-/-}$ mice are protected from renal fibrosis associated with 1K/DOCA/salt-dependent hypertension

Picrosirius red staining revealed that wild-type mice treated with 1K/DOCA/salt had significantly elevated levels of renal interstitial collagen compared with the 1K/placebo-treated control mice (Figure 7). Collagen content in kidneys from 1K/DOCA/salt-treated ASC$^{-/-}$ mice was markedly lower than that in similarly treated wild-type mice (Figure 7).

A specific NLRP3 inhibitor reverses 1K/DOCA/salt-induced increases in systolic BP and reduces renal pro-inflammatory cytokine expression

We evaluated the effects of intervention with a novel, specific inhibitor of NLRP3 inflammasome activity, MCC950, on BP in wild-type mice with established 1K/DOCA/salt-induced hypertension. At day 10 of 1K/DOCA/salt treatment, mice had significantly elevated BP (Figure 8A). Initiation of treatment with MCC950 at this time-point led to a gradual fall in BP such that by day 21, systolic BP had almost returned to baseline (Figure 7). By contrast, treatment with the vehicle for MCC950 did not affect systolic BP in hypertensive mice (Figure 8A). MCC950 treatment also decreased renal expression of the pro-inflammatory cytokines IL-17, TNF and osteopontin, and appeared to reduce IL-6 expression, although this latter effect was not statistically significant (Figure 8B–E).

Discussion

This study highlights the crucial role of inflammasomes in the development of renal inflammation, fibrosis and elevated BP in 1K/DOCA/salt-dependent hypertension in mice. Furthermore, it demonstrates that a small-molecule inhibitor of inflammasome activity is effective at reversing BP and attenuating renal inflammation, even when administered to animals with established hypertension, thus providing proof-of-concept that this may be a viable strategy for the treatment of hypertension.
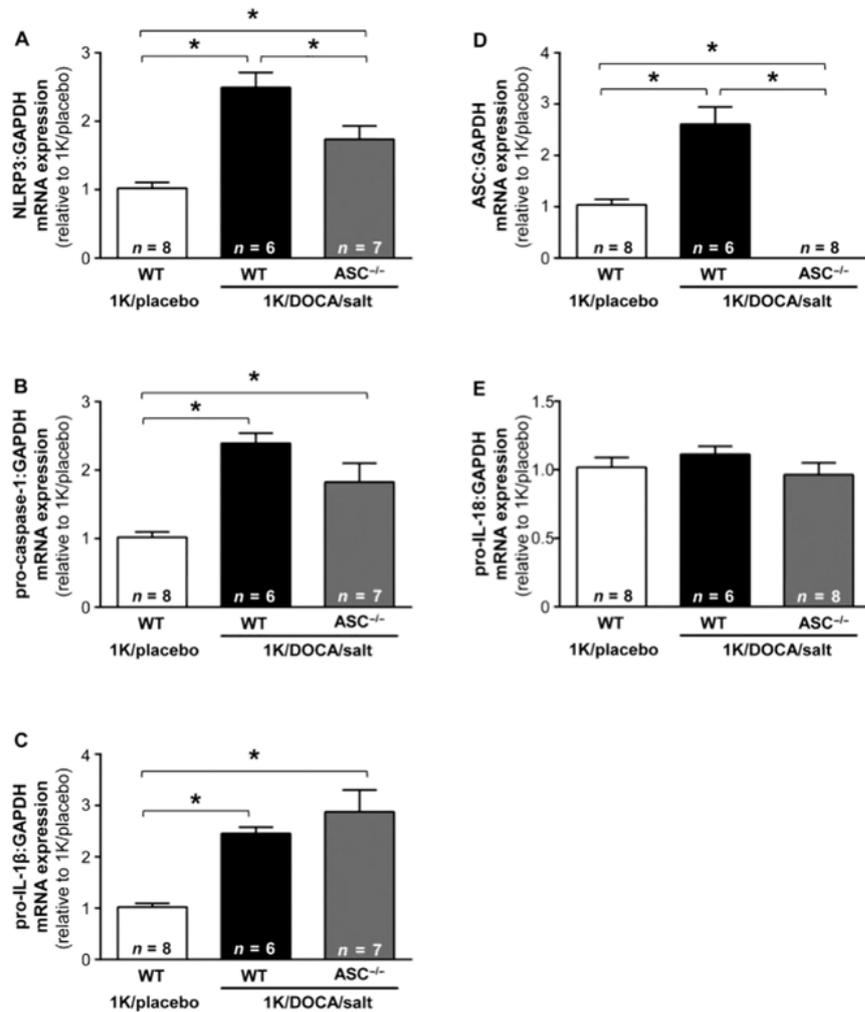
Figure 4

Inflammasome priming in wild-type and ASC$^{-/-}$ mice following treatment with 1K/DOCA/salt. Effects of 1 kidney (1K)/DOCA/salt treatment on renal expression levels of NLR family, pyrin domain containing 3 (NLRP3); (A), pro-caspase-1 (B), pro-IL-1β (C), apoptosis-associated speck-like protein containing a CARD (ASC; D) and pro-IL-18 (E) in wild-type and ASC$^{-/-}$ mice. Values are expressed as mean ± SEM. *$P < 0.05$ for one-way ANOVA followed by Bonferonni's correction for three comparisons.

There is indirect evidence that inflammasome activity plays a role in the development of hypertension in humans. For example, hypertensive patients have elevated circulating levels of IL-1β and IL-18 (Dalekos *et al.*, 1997; Rabkin, 2009; Barbaro *et al.*, 2015) and serum levels of IL-1β are positively correlated with BP (Dalekos *et al.*, 1997). Omi *et al.* (2006) showed that a particular gain-in-function polymorphism in the NLRP3 gene occurs more frequently in hypertensive than in normotensive individuals. In addition, there appears to be a gene-dose effect of the polymorphism on BP, whereby heterozygotes and homozygotes for the polymorphism display 2 and 5 mmHg increases in BP compared with wild-type individuals respectively (Omi *et al.*, 2006). These observations provide a rationale for further examining whether a cause–effect relationship exists between inflammasome activity and hypertension and thus whether inflammasomes might represent targets for novel anti-hypertensive therapies.

Regarding preclinical studies, to our knowledge, there is no published data for increased circulating IL-1β or IL-18 in experimental models of hypertension. Indeed, we too found no evidence for elevated serum levels of these cytokines in mice with 1K/DOCA/salt hypertension. Nonetheless, a recent study showed that chronic treatment with an IL-1β-neutralizing antibody prevented renin-dependent hypertension (two kidneys/one clip) in mice (Wang *et al.*, 2014). These investigators also showed that hypertension was blunted in inflammasome-deficient ASC$^{-/-}$ and NLRP3$^{-/-}$ mice (Wang *et al.*, 2014). Thus, when considered alongside the current findings, these observations highlight the important role of inflammasome-derived IL-1β in the pathogenesis of hypertension.

In further support of a crucial role for inflammasome-derived IL-1β, several studies have shown that cytokines downstream of IL-1β are up-regulated and essential for the

S M Krishnan et al.
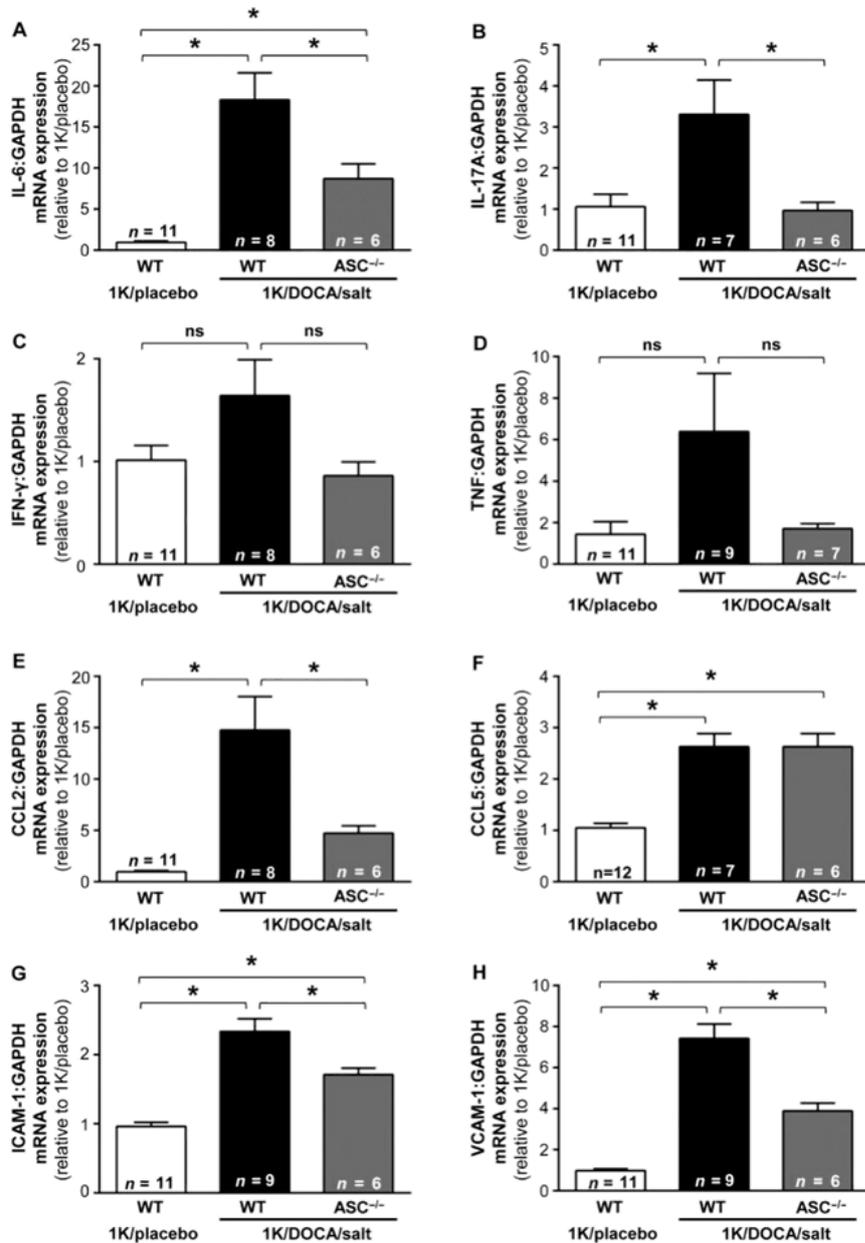
Figure 5

ASC gene deficiency limits 1K/DOCA/salt-induced increases in multiple renal inflammatory markers. Effects of 1 kidney (1K)/DOCA/salt treatment on renal expression levels of IL-6 (A), IL-17A (B), TNF (C), chemokine CCL2 (D), CCL5 (E), ICAM-1 (F) and VCAM-1 (G) in wild-type and ASC$^{-/-}$ mice. Values are expressed as mean ± SEM. *$P < 0.05$, ns, not significant for one-way ANOVA followed by Bonferonni's correction for three comparisons.

development of hypertension in experimental models. IL-6 is released from macrophages and T-cells in response to IL-1β stimulation (Cahill and Rogers, 2008). IL-6 levels are known to be elevated in hypertension (Humbert et al., 1995; Bautista et al., 2005; Lee et al., 2006) and genetic deletion of IL-6 reduces BP, renal injury and fibrosis in angiotensin II-treated mice (Lee et al., 2006; Zhang et al., 2012). IL-1β and IL-18, working in concert with IL-23, can stimulate production of

IL-17A from T-helper 17 and γδ T-cells (Sutton et al., 2009; Lalor et al., 2011), and recent work demonstrated that IL-17A$^{-/-}$ mice are resistant to angiotensin II-dependent hypertension and vascular dysfunction (Madhur et al., 2010). In the present study, we showed that mRNA expression levels of pro-IL-1β, IL-6 and IL-17A, were all elevated in kidneys of wild-type mice following 1K/DOCA/salt treatment. While expression of pro-IL-1β was similarly high in kidneys of
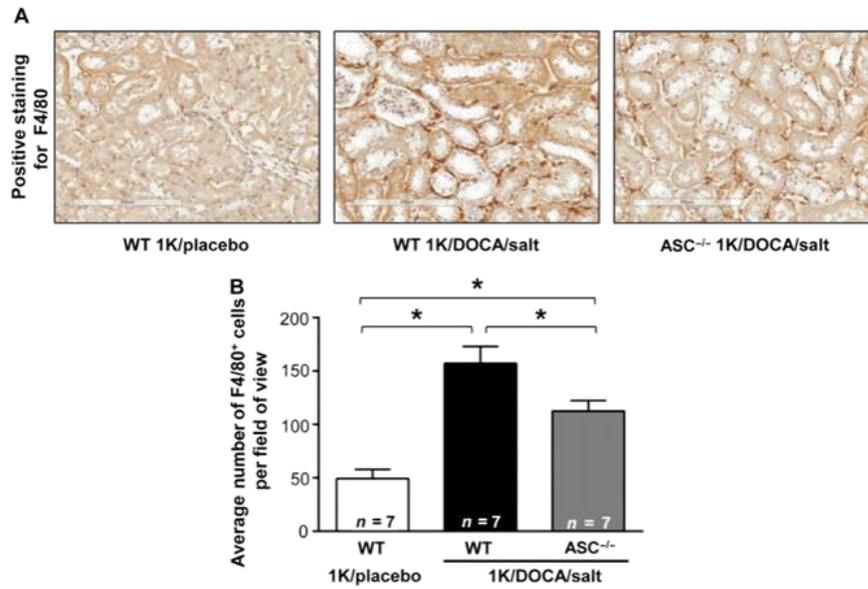
Figure 6
ASC gene deficiency prevents 1K/DOCA/salt-induced increases in macrophage infiltration of the kidney. F4/80 stained kidney sections from normotensive wild-type mice (1K/placebo), and from wild-type and ASC^{−/−} mice following 1 kidney (1K)/DOCA/salt treatment. Upper panels are representative images ($\times 15$ magnification; scale bar = 200 μm) while lower panel shows the quantified group data. Values are expressed as mean ± SEM *$P < 0.05$ for one-way ANOVA followed by Bonferonni's correction for three comparisons.
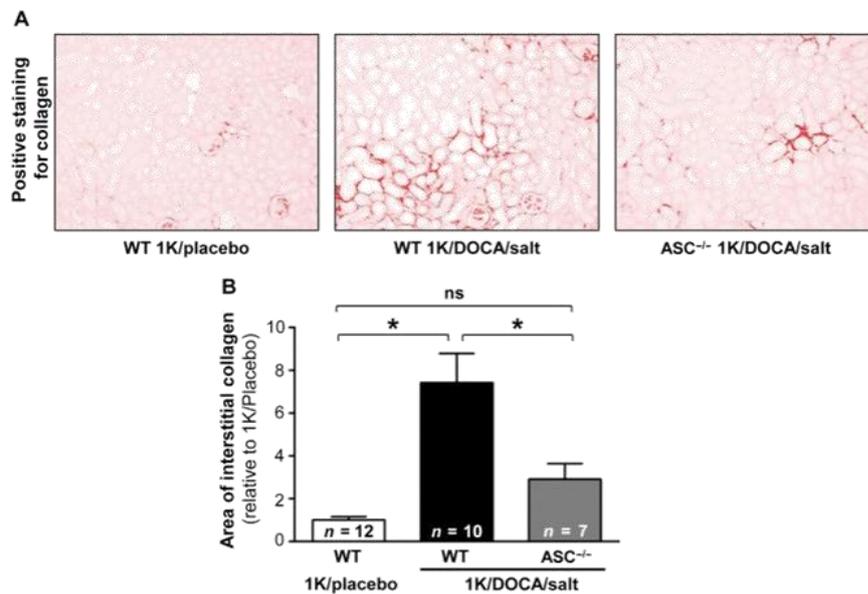
Figure 7
ASC gene deficiency protects mice against 1K/DOCA/salt-dependent renal fibrosis. Picrosirius red-stained kidney sections from normotensive wild-type mice (1K/placebo), and from wild-type and ASC^{−/−} mice following 1 kidney (1K)/DOCA/salt treatment. Upper panels are representative images ($\times 20$ magnification) while lower panel shows the quantified group data. Values are expressed as mean ± SEM. *$P < 0.05$; ns, not significant for one-way ANOVA followed by Bonferonni's correction for three comparisons.

246

S M Krishnan et al.
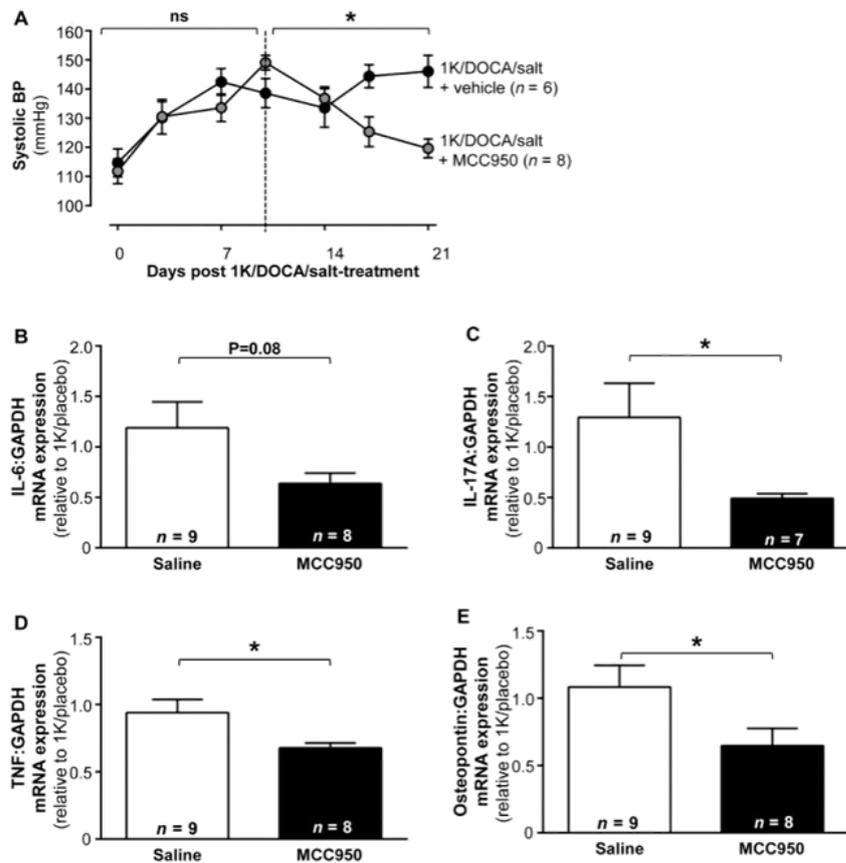
Figure 8

An NLRP3 inhibitor reverses BP and reduces renal expression of pro-inflammatory cytokines in hypertensive mice. Hypertension was induced in mice by 1 kidney (1K)/DOCA/salt treatment and then after 10 days (dotted line) they were further treated with either MCC950 (10 mg·kg^{-1}·day^{-1}, s.c.) or vehicle (0.11 µL·h^{-1}, s.c.) for the remainder of the experiment. Effect of intervention with MCC950 in hypertensive mice on systolic BP (A); and renal mRNA expression of pro-inflammatory cytokines, IL-6 (B), IL-17A (C), TNF (D) and osteopontin (E). All values are expressed as mean ± SEM. *$P \leq 0.05$, ns , not significant for two-way repeated-measures ANOVA before and after the intervention, respectively (A), or Student's unpaired t-test (B–E).

IK/DOCA/salt-treated ASC$^{-/-}$ mice, levels of IL-6 and IL-17A were markedly attenuated. Collectively, these findings are consistent with the trans-activation events leading to increased IL-1β versus IL-6/IL-17A occurring upstream and downstream, respectively, of inflammasome activation.

In addition to effects on IL-6 and IL-17A, ASC$^{-/-}$ deficiency prevented up-regulation of several other inflammatory markers in the kidneys of 1K/DOCA/salt-treated mice including CCL2, ICAM-1 and VCAM-1. These chemokines/adhesion molecules are crucial mediators of leukocyte extravasation and infiltration of tissues in numerous inflammatory conditions, including hypertension (Theuer et al., 2002; Chan et al., 2012). Consistent with such a role, immunohistochemical analysis revealed that ASC$^{-/-}$ mice were also protected from 1K/DOCA/salt-induced macrophage accumulation in the kidneys.

Of note, we found no evidence of elevated IL-18 levels, either in kidneys or blood of 1K/DOCA/salt-treated mice. Moreover, expression of IFN-γ, a cytokine activated directly downstream of IL-18 (Dinarello, 1999), was also unchanged in kidneys from 1K/DOCA/salt versus control animals. Hence, the protective effects of inflammasome inhibition against hypertension are likely due to inhibition of IL-1β rather than IL-18 signalling pathways.

Inflammasome activity is regulated at two levels. First, the various components of the NLRP3 inflammasome complex and cytokine precursors are normally expressed at low levels, but undergo transcriptional upregulation or 'priming' following stimulation of the transcription factor NF-κB (Bauernfeind et al., 2009). Here we observed that 1K/DOCA/salt-induced hypertension in mice was associated with increased mRNA levels of NLRP3, ASC, pro-caspase-1 and pro-IL-1β, indicating priming of the system. Previous studies have shown that 1K/DOCA/salt-dependent hypertension is associated with increased NF-κB activity in the kidneys (Beswick et al., 2001; Elmarakby et al., 2008). Moreover, pro-hypertensive stimuli such as aldosterone and angiotensin II can directly increase NF-κB activity in myeloid cells

(Chantong *et al.*, 2012; Lee *et al.*, 2014). Hence, we would suggest that these agonists represent strong candidates as the stimuli responsible for inflammasome priming in the present study.

In addition to priming, inflammasomes must undergo assembly to promote auto-cleavage and activation of pro-caspase-1 and facilitate processing of IL-1β and/or IL-18 (Latz, 2010). Thus, protein expression levels of caspase-1 p10/20 and IL-1β p17 subunits provide measures of inflammasome activation (Mariathasan *et al.*, 2006; Hornung *et al.*, 2008). Here we used Western blotting and commercially available antibodies to measure caspase-1 p10 and IL-1β p17 subunits, and in both instances observed multiple immunoreactive bands. While the poor specificity of the antibodies is clearly a limitation of our study, based on the negative control sample (kidney proteins from caspase-1$^{-/-}$ mice) and predicted molecular weights of the cleaved proteins, it would appear that 1K/DOCA/salt-induced hypertension is associated with inflammasome activation in the kidneys. Assembly of the inflammasome complex occurs in response to DAMPs including microcrystals, protein aggregates and high extracellular ATP (Mariathasan *et al.*, 2006; Martinon *et al.*, 2006; Halle *et al.*, 2008). At present, we have no evidence that 1K/DOCA/salt-dependent hypertension is associated with increases in any of these DAMPs. However, it is noteworthy that even in control animals, we were able to detect low levels of both caspase-1 p10 and IL-1β p17 in the kidneys, suggesting that priming alone may have been sufficient to account for the increase in active caspase-1 and IL-1β in hypertensive versus normotensive animals. Indeed, this is supported by our observation that the ratio of cleaved : uncleaved caspase-1 was not elevated in hypertensive versus normotensive animals.

Although our data indicate that priming may be more important for increased IL-1β signalling in the 1K/DOCA/salt model, this does not mean that stimuli that promote inflammasome assembly could not contribute to hypertension in other settings. For example, gout and hyperuricaemia are associated with formation of urate microcrystals at multiple sites, including the kidneys, resulting in inflammasome activation (Wu *et al.*, 1994; Martinon *et al.*, 2006). The link between hyperuricaemia/gout and hypertension has been established for over a century (Haig, 1889; Bos *et al.*, 2006), and treatment with urate-lowering compounds such as allopurinol lowers BP in a large proportion of individuals (Feig *et al.*, 2008). More recently, Mazzali *et al.* (2001) showed that induction of mild hyperuricemia in rats causes them to become hypertensive, further suggesting a causal link between the two conditions. Together with our new data, these observations raise the possibility that increased inflammasome activity in the kidneys, either resulting from priming and/or activation, may be a fundamental mechanism in the development of hypertension.

Our finding that ASC deficiency afforded protection against the chronic pressor, renal inflammatory and pro-fibrotic actions of 1K/DOCA/salt, and the hypertensive effects of angiotensin II, implies that targeting the inflammasome/IL-1β nexus may be a new approach for reducing BP and renal damage in hypertension. There are currently three drugs in clinical use for the treatment of autoimmune diseases such as rheumatoid arthritis and gout that target IL-1β signalling including: anakinra, a recombinant human IL-1 receptor antagonist; rilonacept, a fusion protein that binds and neutralizes IL-1β; and canakinumab, a human monoclonal IL-1β neutralizing antibody (Mertens and Singh, 2009; Schlesinger, 2014). A clinical trial is currently underway to evaluate canakinumab as a treatment for cardiovascular disease (Ridker *et al.*, 2011); however, there is currently no clinical data on potential anti-hypertensive effects of these aforementioned IL-1β inhibitors. Recently, a novel small-molecule inhibitor of NLRP3 activity, MCC950, was described (Coll *et al.*, 2015) with an IC$_{50}$ of 7.5–8.0 nM *in vitro* and efficacy *in vivo* in an experimental model of multiple sclerosis (Coll *et al.*, 2015). We now show that MCC950 is similarly effective at reducing BP and expression of several markers of renal inflammation and damage, even when administered after the establishment of hypertension in mice. Together with the findings of Wang *et al.* (2014), these data provide proof-of-concept that pharmacological inhibition of the inflammasome/IL-1β pathway is likely to be an effective strategy for the treatment of hypertension.

In conclusion, we have provided evidence that increased inflammasome activity in the kidney, leading to IL-1β maturation and activation of downstream pro-inflammatory pathways, plays a key role in the pathogenesis of hypertension. These findings add to the growing body of evidence suggesting that hypertension is an inflammatory disease, and also highlight new possibilities for therapies to treat hypertension and its sequelae of renal disease by specifically targeting inflammasome/IL-1β signalling.

Author contributions

S. M. K., J. K. D., H. D., D. F., A. P. and T. D. H. performed the research. S. M. K., E. L., A. M., C. G. S. and G. R. D. designed the research study. A. A. B. R., M. A. C., A. M. contributed essential reagents or tools. S. M. K., T. D. H. and G. R. D. analysed the data. S. M. K., Y. H. L., C. T. C., M. M. K., C. S. S., A. V., T. V. A., B. K. K-H., E. L., A. M., C. G. S. and G. R. D. wrote the paper.

Appendix

Conflict of interest

The authors have no competing interests.

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250

ORIGINAL PAPER

Vitamin D₃ Supplementation Reduces Subsequent Brain Injury and Inflammation Associated with Ischemic Stroke

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Abstract

Acute inflammation can exacerbate brain injury after ischemic stroke. Beyond its well-characterized role in calcium metabolism, it is becoming increasingly appreciated that the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25-VitD₃), has potent immunomodulatory properties. Here, we aimed to determine whether 1,25-VitD₃ supplementation could reduce subsequent brain injury and associated inflammation after ischemic stroke. Male C57Bl6 mice were randomly assigned to be administered either 1,25-VitD₃ (100 ng/kg/day) or vehicle i.p. for 5 day prior to stroke. Stroke was induced via middle cerebral artery occlusion for 1 h followed by 23 h reperfusion. At 24 h post-stroke, we assessed infarct volume, functional deficit, expression of inflammatory mediators and numbers of infiltrating immune cells. Supplementation with 1,25-VitD₃ reduced infarct volume by 50% compared to vehicle. Expression of pro-inflammatory mediators IL-6, IL-1β, IL-23a, TGF-β and NADPH oxidase-2 was reduced in brains of mice that received 1,25-VitD₃ versus vehicle. Brain expression of the T regulatory cell marker, Foxp3, was higher in mice supplemented with 1,25-VitD₃ versus vehicle, while expression of the transcription factor, ROR-γ, was decreased, suggestive of a reduced Th17/γδ T cell response. Immunohistochemistry indicated that similar numbers of neutrophils and T cells were present in the ischemic hemispheres of 1,25-VitD₃- and vehicle-supplemented mice. At this early time point, there were also no differences in the impairment of motor function. These data indicate that prior administration of exogenous vitamin D, even to vitamin D-replete mice, can attenuate infarct development and exert acute anti-inflammatory actions in the ischemic and reperfused brain.

Keywords Vitamin D · Inflammation · Stroke · Middle cerebral artery occlusion · Mouse

Introduction

Stroke is the world's second leading cause of death, contributing to 6.7 million deaths annually (Mozaffarian et al. 2016). It is also the most frequent cause of permanent

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disability in adults, with half of all survivors discharged into care (Mozaffarian et al. 2016). Currently, there is only one approved pharmacological agent available to treat stroke, recombinant tissue plasminogen activator (rtPA), which must be administered within a 4.5-h window of stroke onset and only after a CT scan has diagnosed a thrombotic cause (Del Zoppo et al. 2009). Due to these strict limitations, < 10% of stroke patients are eligible to receive rtPA (Reeves et al. 2005; Kleindorfer et al. 2008). Consequently, there is a desperate need to identify modifiable mechanisms capable of limiting the impact of acute stroke.

Secondary brain injury following stroke is driven by local inflammation, production of reactive oxygen species and the infiltration of circulating immune cells (Anrather and Iadecola 2016). Thus, targeting these inflammatory processes has been of intense interest to stroke researchers. However, one immunomodulatory molecule that has received very little attention as a potential stroke therapy is vitamin D, a fat-soluble vitamin that functions as a steroid hormone. Vitamin D is synthesized predominantly from 7-dehydrocholesterol in response to skin exposure to ultraviolet light, but can also be obtained through dietary supplementation (Holick 2007). To become biologically active, vitamin D must first be converted to 1,25-dihydroxyvitamin D_3 (1,25-VitD$_3$) via two hydroxylation steps. This occurs firstly in the liver by 25-hydroxylase and then typically in the kidney by 1-α-hydroxylase (CYP27B) (Holick 2007). The latter hydroxylation step can also occur in macrophages, T cells and neurons, which also express 1-α-hydroxylase (Lugg et al. 2015). Once in this active form, vitamin D can engage with the vitamin D receptor (VDR) which is located on a number of cell types including leukocytes, endothelial cells, astrocytes and neurons (Provvedini et al. 1983; Merke et al. 1989; Langub et al. 2001; Lee et al. 2008). Vitamin D is best characterized to promote calcium absorption from the small intestine, but recent findings indicate that it may also control expression of a large number of genes, particularly those involved in inflammatory processes (Lugg et al. 2015).

1,25-VitD$_3$ exerts such immunomodulatory actions through a variety of cellular and molecular mechanisms. Firstly, 1,25-VitD$_3$ can prevent the development of pathogenic T helper (Th) 1, Th17 and γδ T cells, and can promote the formation of anti-inflammatory Th2 and T regulatory cells (Zeitelhofer et al. 2017; Chang et al. 2010a; Gregori et al. 2002; Joshi et al. 2011; Nashold et al. 2013; Sloka et al. 2011; Cantorna et al. 2004; Hart et al. 2011; Chen et al. 2005). Studies have also shown that 1,25-VitD$_3$ promotes the generation of tolerogenic dendritic cells (Takeda et al. 2010; Gorman et al. 2010) and can prevent the release of pro-inflammatory cytokines from monocytes and microglia (Korf et al. 2012; Zhang et al. 2012; Boontanrart et al. 2016; Verma and Kim 2016). Further, 1,25-VitD$_3$ may inhibit the production of reactive oxygen species by decreasing

expression of NADPH oxidase (NOX) enzymes (Dong et al. 2012) and enhancing expression of antioxidants such as superoxide dismutase and glutathione (Jain and Micinski 2013; Dong et al. 2012).

Observational studies have documented that patients with lower serum levels of vitamin D experience larger infarct volumes and worse functional outcomes following stroke (Tu et al. 2014; Wang et al. 2014; Turetsky et al. 2015; Daubail et al. 2013; Park et al. 2015), suggesting that vitamin D may play a protective role during cerebral ischemia. We recently reported that low baseline levels of vitamin D, resulting from a vitamin D-deficient diet, had no discernible impact on selected outcome measures within 24 h of large vessel occlusion stroke (Evans et al. 2017). Here, we have instead examined the effect of elevated baseline levels of vitamin D achieved by supraphysiological doses of vitamin D given to vitamin D-replete animals during the 5 days prior to stroke, in an analogous manner to high dose supplementation regimes in humans (Wong et al. 2014; Sotirchos et al. 2016). For this, we adopted a similar supplementation regime that was found to reduce vascular injury in mice following hindlimb ischemia (Wong et al. 2014). Indeed, we report that 1,25-VitD$_3$ supplementation can reduce post-stroke brain injury, reduce expression of pro-inflammatory cytokines, modulate the phenotype of T cells and increase the number of M2-polarized (anti-inflammatory) macrophages/microglia in the brain.

Materials and Methods

Animals

A total of 92 male C57Bl6 mice (7–10 week old; 21–30 g) were used for this study. Mice were housed under a 12-h light/dark cycle and had free access to water and food pellets. Mice were excluded from the study if during the surgical procedure to induce middle cerebral artery occlusion: [1] > 0.2 ml of blood was lost (n = 1); [2] subarachnoid hemorrhage occurred (n = 2); [3] death occurred during ischemia (n = 2); [4] cerebral blood flow failed to reach ≥ 80% pre-ischemic levels upon reperfusion (n = 1) and [5] death occurred after reperfusion and prior to the designated time for euthanasia (n = 7). All animals were randomly assigned to groups, and the investigator performing the surgical procedure or data analysis was, wherever possible, blinded to the treatment group.

Administration of 1-25,Dihydroxyvitamin D$_3$

Vitamin D$_3$ was administered as its active form, 1α, 25-dihydroxyvitamin D$_3$ (1,25-VitD$_3$; Sigma; 100 ng/kg/day) which was dissolved in a solvent mixture of sterile water, propylene

glycol and ethanol in a 5:4:1 ratio. Animals were injected i.p. for 5 consecutive days prior to experimental stroke and again on the day of the procedure, as previously described (Wong et al. 2014).

Middle Cerebral Artery Occlusion

Mice underwent either sham surgery or focal cerebral ischemia as previously described (Evans et al. 2017). Cerebral ischemia was produced in anesthetized mice (ketamine: 80 mg/kg plus xylazine: 10 mg/kg i.p.) by occlusion of the middle cerebral artery (MCA) using a 6.0 silicone-coated monofilament (Doccol Corporation). Rectal temperature was monitored and maintained at 37.0 ± 0.5 °C. MCA occlusion (MCAO) was sustained for 60 min, and the filament then retracted to allow reperfusion. Both successful occlusion (> 70% reduction in cerebral blood flow; CBF) and reperfusion ($\geq$ 80% return of CBF to the pre-ischemic level) were confirmed by transcranial laser-Doppler flowmetry (PeriMed). Sham-operated mice were anesthetized and the right carotid bifurcation exposed, but no filament was inserted. Neck wounds were then closed with sutures and covered with Betadine® (Sanofi) and spray dressing. Head wounds were closed with superglue, and mice were returned to their cages after regaining consciousness.

Functional Assessment

Mice were assessed for functional deficits at approximately 30 min prior to euthanasia. This comprised a 6-point scoring system for neurological deficits: 0 = normal motor function, 1 = flexion of torso and contralateral forelimb when lifted by the tail, 2 = circling to the contralateral side when held by the tail on a flat surface but normal posture at rest, 3 = leaning to the contralateral side at rest, 4 = no spontaneous motor activity, 5 = death. A hanging grip test was performed as a measure of grasping ability and forelimb strength in which mice were suspended by their forelimbs on a wire between 2 posts 60 cm above a soft pillow for up to 60 s. The time until the animal fell was recorded (a score of 0 s was assigned to animals that fell immediately and a score of 60 s was assigned to animals that did not fall), and the average time of 3 trials with 5 min rests in between was calculated. Spontaneous locomotor activity was assessed using a parallel rod floor apparatus using ANY-maze software coupled to an automated video-tracking system as previously described (Lee et al. 2015).

Assessment of Infarct Volume

Cerebral infarct volumes were determined as previously described (Evans et al. 2017). At 24 h post-stroke, mice were killed by inhalation of isoflurane followed by decapitation. Brains were immediately removed, snap-frozen in liquid nitrogen and stored at − 80 °C. Evenly spread (separated by ~ 420 μm) coronal sections (30 μm) spanning the infarct were cut, thaw-mounted onto poly-L-lysine coated glass slides and stained with 0.1% thionin (Sigma) to delineate the infarct area. Infarct volume was quantified using image analysis software (ImageJ, NIH), correcting for brain edema, according to the following formula: $CIV = (LHA - (RHA - RIA)) \times$ (thickness of section + distance between sections); where CIV is corrected infarct volume, LHA is left hemisphere area, RHA is right hemisphere area and RIA is right hemisphere infarct area. Edema-corrected infarct volumes of individual brain sections were then added, giving an approximation of the total infarct volume.

Real-Time Polymerase Chain Reaction (rt-PCR)

At 24 h following stroke or sham surgery, mice were euthanized by isoflurane overdose and perfused with RNase-free phosphate-buffered saline (PBS). After removing the cerebellum and olfactory bulbs, the brain was separated into left and right hemispheres and snap-frozen in liquid nitrogen for RNA extraction. Spleens were also removed, cut in half and snap-frozen in liquid nitrogen. Tissues were stored at − 80 °C until required. Total RNA was extracted using Qiazol® reagent (Qiagen) and the RNeasy Mini Kit with on-column DNase step (Qiagen) followed by cDNA conversion using the Quantitect Reverse Transcription kit (for Taqman® gene expression assays; Qiagen). The cDNA was then used as a template in real-time PCR to measure mRNA expression of *Vdr, Cyp27b, Cyp24a, Cxcl12, Tbx21, Stat4, Rorc, Gata3, Stat6, Foxp3, Tnfα, Il1β, Il6, Il21, Il23a, Tgfβ1, Ccl2, Ccl5, Gp91phox, Mrc1, Il10* and *Icam1. Gapdh* and *β-actin* were assessed as housekeeping genes for brain and spleen tissue, respectively. Assays were performed according to the manufacturer's instructions using the Bio-Rad CFX96TM real-time PCR machine (Bio-Rad). Data were normalized to the housekeeping gene and calculated as change in fold expression relative to sham using the formula: fold-change = $2^{-\Delta\Delta Ct}$.

Immunofluorescence

Six serial coronal sections (10 μm thick) per animal were collected at six regions: bregma + 0.06, − 0.78, − 1.2, − 1.62, − 2.04, − 2.46 mm. Frozen brain sections (10 μm) were fixed in 4% paraformaldehyde for 15 min and washed in 0.01 M PBS (3 × 10 min). Sections were then blocked with 10% goat serum (Sigma) for 60 min to block non-specific binding of the secondary antibody. Sections were then incubated overnight at 4 °C with either rabbit anti-CD3 (1:200; Abcam) or rabbit anti-CD206 (1:500; Abcam). On

the following day, they were washed (PBS; 3 × 10 min) and incubated for a maximum of 2 h with either goat anti-rabbit Alexa Fluor 594 (1:500; Thermofisher Scientific) or goat anti-rabbit Alexa Fluor 488 (1:500; Thermofisher Scientific). Finally, sections were again washed and then mounted with Vectashield medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories), and a coverslip was applied. All tissue-mounted slides were viewed, analyzed and photographed with an Olympus fluorescence microscope. Numbers of immunoreactive cells were counted manually per whole ischemic hemisphere and then averaged across the six regions, as indicated above.

3,3′-Diaminobenzidine (DAB) Immunohistochemistry

Frozen brain sections (at the regions indicated for immunofluorescence) were fixed in 4% paraformaldehyde for 15 min, washed in PBS and then incubated in peroxidase blocking solution (Dako) for 10 min to block endogenous peroxidases followed by 10% goat serum for 60 min. They were then incubated overnight at room temperature in rabbit anti-myeloperoxidase (1:100; Abcam). The following day, sections were washed and incubated for 2 h in anti-rabbit IgG horse-radish peroxidase conjugate (1:200; Dako), washed again, and DAB (Dako) was then applied for 5–10 min. Sections were then rinsed in dH$_2$O, dehydrated in increasing concentrations of ethanol (70 and 100% vol/vol), cleared in xylene and mounted in DPX. Tissue-mounted slides were viewed, analyzed and photographed using an Olympus light microscope. Numbers of immunoreactive cells were counted manually per whole ischemic hemisphere and then averaged across the six regions, as indicated above.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM), with the exception of neurological deficit scores, which are presented as median. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc. San Diego, CA, USA). Between-group comparisons were compared using one-way ANOVA, or Student's unpaired t test, as appropriate. If differences were detected by ANOVA, individual groups were compared with Tukey's multiple comparisons test, where indicated. Neurological deficit scores were compared using a Kruskal–Wallis test followed by Dunn's multiple comparisons test. If there were two independent variables, data were compared using a two-way ANOVA. Statistical significance was accepted if $P < 0.05$.

Results

Effect of Cerebral Ischemia on Vitamin D-Associated Genes in Brain and Spleen

The effect of stroke was first assessed on the expression of VDR and metabolizing enzymes in the brain and spleen at 24 h in otherwise untreated animals. Stroke increased expression of the VDR by ~twofold in both organs (Fig. 1a, b). The vitamin D-activating enzyme, *Cyp27b*, was reduced by ~ 30% in the brain, but was unchanged in the spleen following stroke (Fig. 1a, b). Expression of the inactivating enzyme, *Cyp24a*, was increased by ~ 2.5 fold in the brain, but was undetectable in spleen (Fig. 1a, b).

Effects of Vitamin D$_3$ Supplementation on Infarct Volume and Functional Deficits Following Stroke

To determine the effect of elevated baseline vitamin D prior to stroke on the extent of subsequent infarct development, mice were treated with 1,25-VitD$_3$ (100 ng/kg/day)
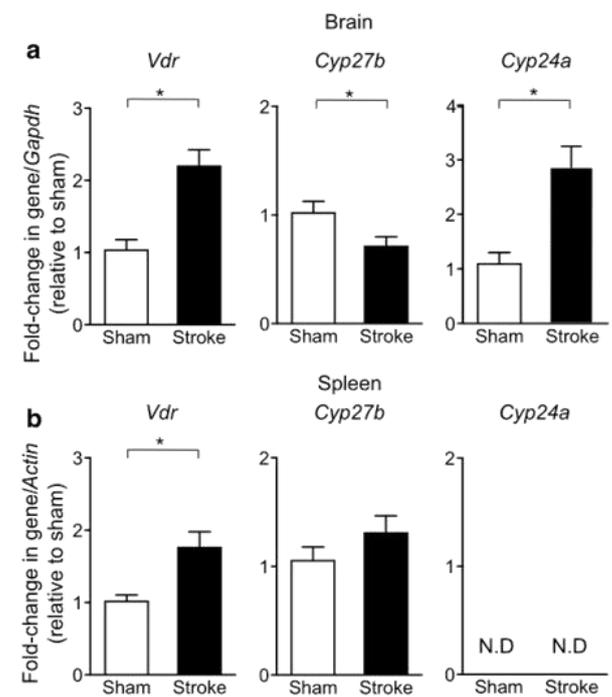

Fig. 1 Post-stroke expression of vitamin D-associated genes. mRNA expression of the vitamin D receptor (*Vdr*), 1-α-hydroxylase (*Cyp27b*) and 24-hydroxylase (*Cyp24a*) was examined in **a** the brain and **b** the spleen at 24 h after either stroke or sham surgery. Sham: $n = 6$–9 per group and stroke: $n = 8$–12 per group. *N.D.* denotes not detected. *$P < 0.05$, Student's unpaired t test. Data are presented as mean ± SEM

for 5 days and then subjected to focal cerebral ischemia. At 24 h post-stroke, we found that mice which received 1,25-VitD$_3$ supplementation had ~ 50% smaller infarct volume than those which received vehicle (Fig. 2a, b). This finding was not associated with any differences in the level of cerebral blood flow during, or immediately after, cerebral ischemia (Fig. S1). Examining the distribution of the infarcts, 1,25-VitD$_3$-supplemented animals tended to have a reduced infarct area in most coronal sections (Fig. 2c).

However, mice in both groups displayed similar functional deficits at this early time point (Fig. 3a–e).

Effect of Vitamin D$_3$ Supplementation on T Cell Phenotype in the Brain and Spleen After Stroke

Previous reports suggest that Th1 and γδ T cells exacerbate brain injury following stroke while Th2 and T regulatory cells play a protective role by dampening excessive inflammation (Gu et al. 2012; Gelderblom et al. 2012; Benakis
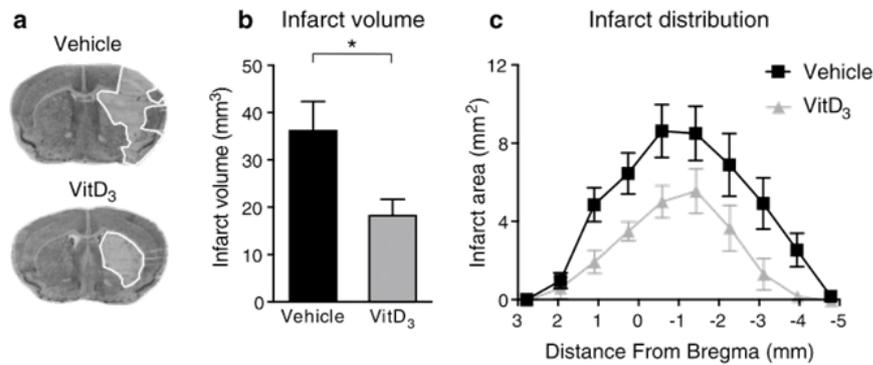
Fig. 2 1,25-Dihydroxyvitamin D$_3$ (VitD$_3$) treatment reduces infarct development. **a** Representative coronal brain sections from vehicle- and VitD$_3$-treated animals (infarct area is outline in white). **b** Infarct volume and **c** distribution of infarct area from vehicle- and VitD$_3$-treated animals. Vehicle: $n = 14$ per group and VitD$_3$: $n = 12$ per group. *$P < 0.05$, Student's unpaired t test, where appropriate. Data are presented as mean ± SEM

Fig. 3 1,25-Dihydroxyvitamin D$_3$ (VitD$_3$) treatment does not influence functional outcome at 24 h following stroke. **a** Neurological deficit scores and **b** latency to fall on hanging grip test of sham, vehicle- and VitD$_3$-treated animals. Parallel rod floor test examining **c** total distance travelled, **d** percentage time mobile and **e** average speed of sham, vehicle- and VitD$_3$-treated animals. Sham: $n = 6$–8 per group, vehicle: $n = 14$ per group and VitD$_3$: $n = 11$–14 per group. **$P < 0.01$, ***$P < 0.001$, Kruskal–Wallis test followed by Dunn's multiple comparisons test (**a**) or one-way ANOVA followed by Tukey's multiple comparison test (**b–e**). Data are presented as mean ± SEM, with the exception of neurological deficit scores which are presented as median

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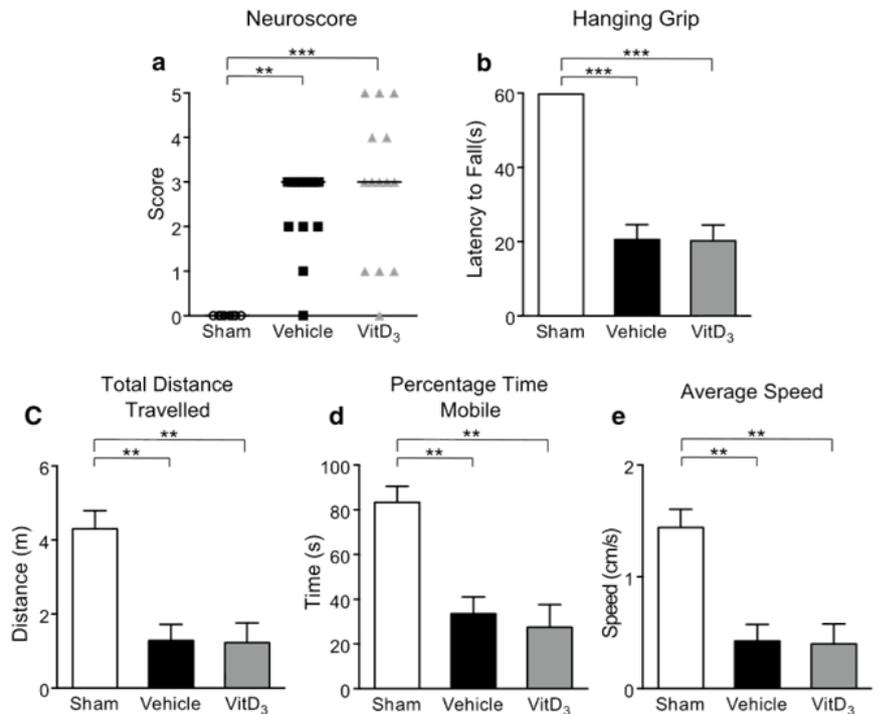
Fig. 4 1,25-Dihydroxyvitamin D$_3$ (VitD$_3$) supplementation alters mRNA expression of T cell transcription factors in the brain and spleen following stroke. **a** Expression of Th1 transcription factors, *Tbx21* and *Stat4*, and Th17 transcription factor, *Rorc*, within the brain of sham, vehicle- (Veh) and VitD$_3$-treated mice at 24 h post-surgery. **b** Expression of Th2 transcription factors, *Gata3* and *Stat6*, and T regulatory cell transcription factor, *Foxp3*, within the brain of sham, vehicle- and VitD$_3$-treated mice at 24 h post-surgery. **c** Expression of *Tbx21*, *Stat4*, and *Rorc* within the spleen of sham, vehicle- and VitD$_3$-treated mice at 24 h post-surgery. **d** Expression of *Gata3*, *Stat6* and *Foxp3* within the spleen of sham, vehicle- and VitD$_3$-treated mice 24 h post-surgery. Sham: $n = 6$–9 per group, vehicle: $n = 11$–13 per group, VitD$_3$: $n = 10$–12 per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as mean ± SEM
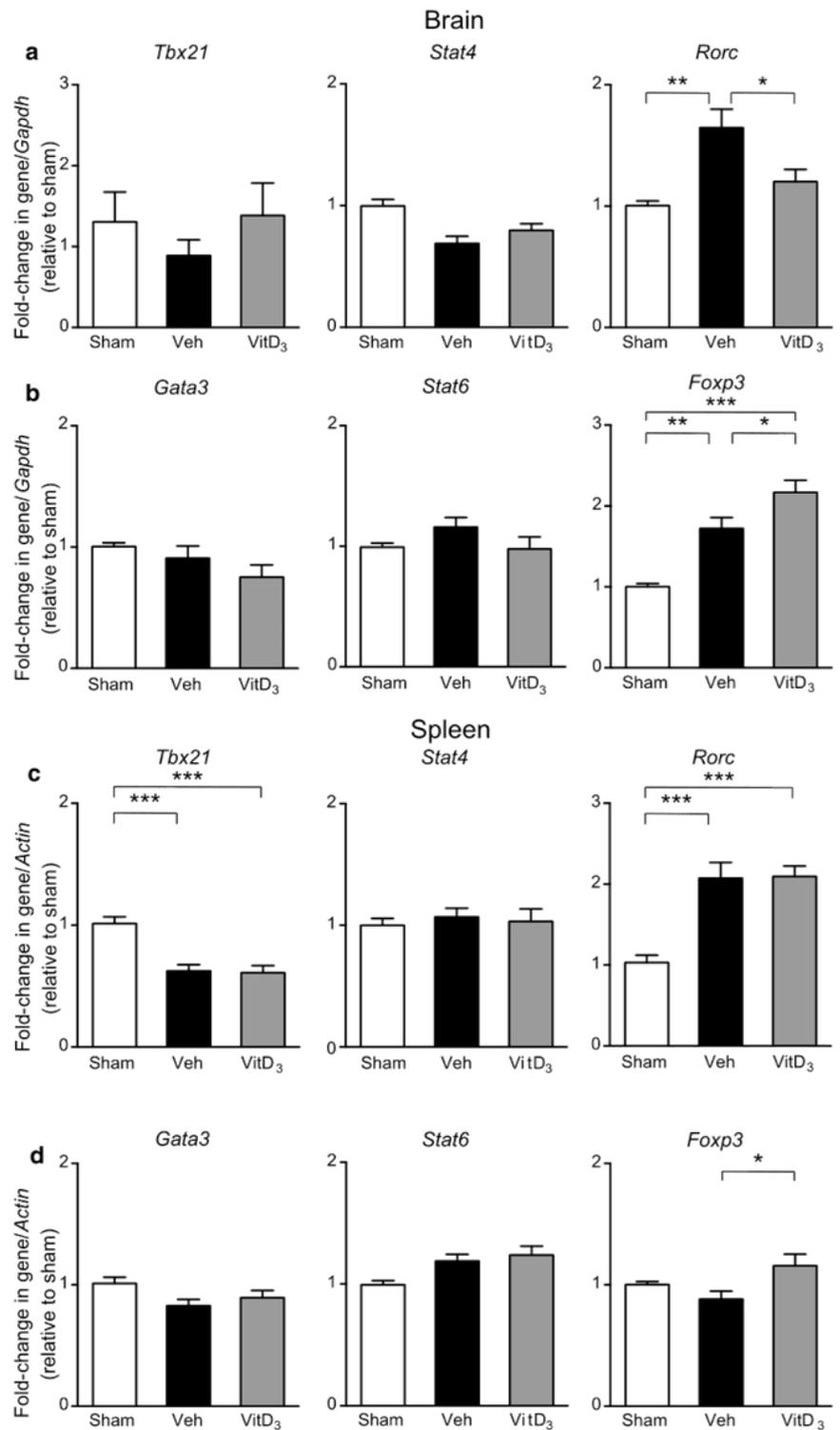
et al. 2016; Liesz et al. 2009). It has been shown that vitamin D can modulate the immune response to injury by polarizing T cells toward an anti-inflammatory phenotype (Hart et al. 2011). We found no effect of stroke or 1,25-VitD$_3$ on mRNA expression of Th1 transcription factors, *Tbx21* or *Stat4*, or Th2 transcription factors, *Gata3* or *Stat6* in the brain

(Fig. 4a, b). However, stroke resulted in an elevation of the Th17/γδ T cell transcription factor, *Rorc*, and this effect was mitigated by 1,25-VitD$_3$ treatment (Fig. 4a). Moreover, we noted an increase in the T regulatory cell transcription factor, *Foxp3*, after stroke, and this was augmented in 1,25-VitD$_3$-supplemented animals (Fig. 4b). In the spleen, 1,25-VitD$_3$ had no effect on expression of *Tbx21*, *Stat4*, *Gata3*, *Stat6* or *Rorc* (Fig. 4c, d). However, *Foxp3* expression was slightly higher after stroke in 1,25-VitD$_3$-treated mice than in sham mice or in those treated with vehicle (Fig. 4d).

Effect of Vitamin D$_3$ Supplementation on Expression of Pro-inflammatory Mediators in the Brain Following Stroke

As vitamin D$_3$ has immunomodulatory actions, we also examined mRNA expression of various inflammatory mediators known to be involved in ischemic brain injury. Indeed, 1,25-VitD$_3$-treated animals had lower expression of *Ilβ*, *Il6*, *Il23a*, *Tgfβ1* and *Gp91phox* (NOX-2) than vehicle-treated animals (Fig. 5b, c, e, f, i). However, there was no effect of

Fig. 5 1,25-Dihydroxyvitamin D$_3$ (VitD$_3$) supplementation alters mRNA expression of inflammatory mediators in the brain following stroke. mRNA expression of **a** *Tnfα*, **b** *Il1β*, **c** *Il6*, **d** *Il21*, **e** *Il23a*, **f** *Tgfβ1*, **g** *Ccl2*, **h** *Ccl5*, **i** *Gp91phox*, **j** *Mrc1*, **k** *Icam1* and **l** *Il10* within the brains of sham, vehicle (Veh)- and VitD$_3$-treated mice at 24 h post-surgery. Sham: $n = 6$–7 per group, vehicle: $n = 12$ per group and VitD$_3$: $n = 9$–11 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as mean $\pm$ SEM
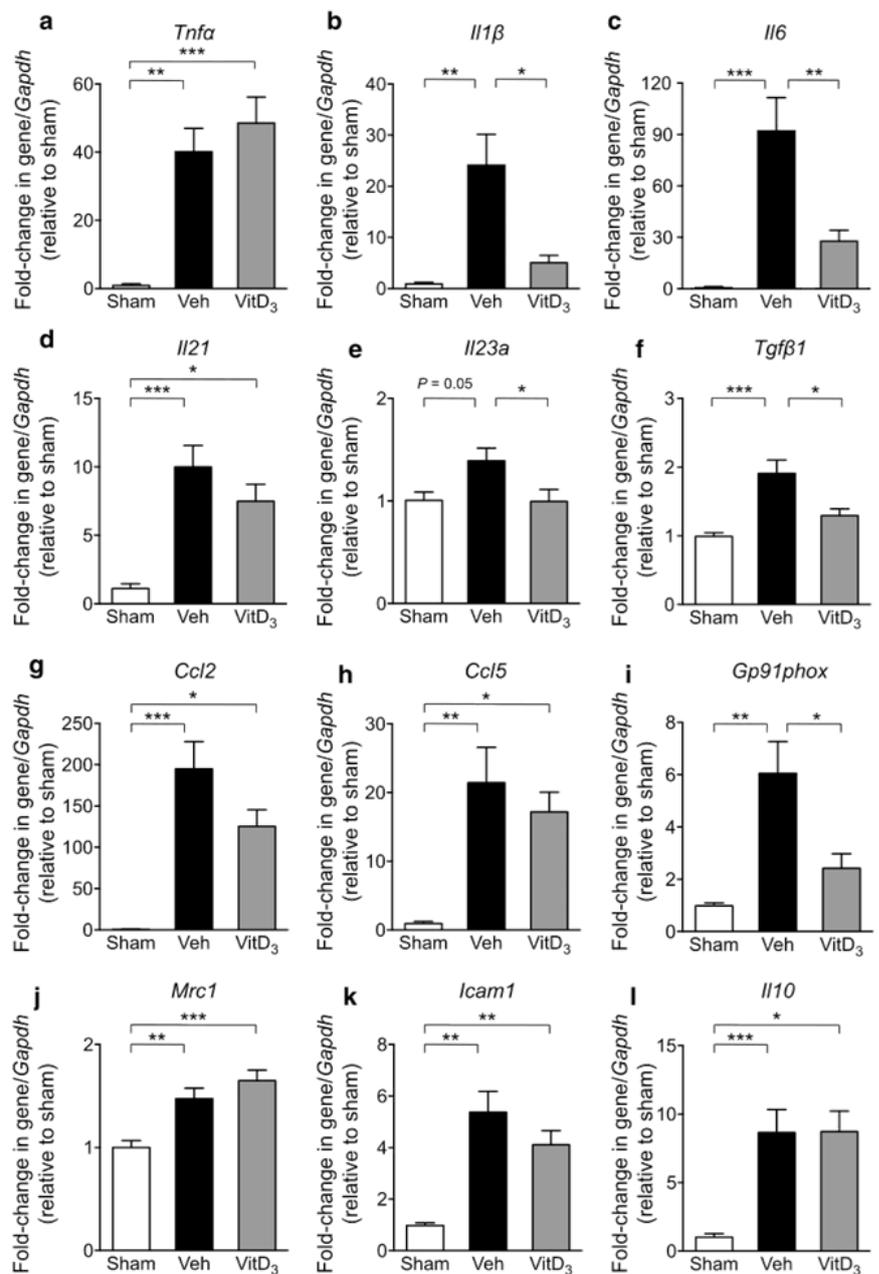
1,25-VitD$_3$ on *Tnfα*, *Il21*, *Ccl2*, *Ccl5*, *Mrc1*, *Icam1* or *Il10* (Fig. 5a, d, g, h, j, k, l).

Effect of Vitamin D$_3$ Supplementation on Numbers of Infiltrating Leukocytes and M2-Polarized Macrophages/Microglia in the Brain Following Stroke

We tested for any effect of vitamin D on migration of immune cells toward the site of injury, by quantifying leukocyte infiltration into the ischemic hemisphere using immunohistochemistry. We noted a tendency for 1,25-VitD$_3$-treated animals to have fewer MPO$^+$ neutrophils in the brain at 24 h post-stroke, whereas there was no effect on CD3$^+$ T cells (Fig. 6a, b). Additionally, there was a trend for greater numbers of "M2" polarized microglia/macrophages (defined as CD206$^+$) after stroke in 1,25-VitD$_3$-treated animals (Fig. 6c).

Discussion

Inflammation is a major contributor to secondary brain injury after ischemic stroke and thus represents a potential target for therapy (Anrather and Iadecola 2016). Beyond its well-characterized role in calcium metabolism, vitamin D has potent immunomodulatory properties and can alter the immune response to injury in various disease settings (Nashold et al. 2013; Takeda et al. 2010; Martorell et al. 2016; Schedel et al. 2016). If vitamin D was found to exert such effects in post-stroke brain injury, it could represent a novel direction for acute therapy. Indeed, here we report data supporting this concept. This neuroprotective effect appears to occur in association with reduced expression of pro-inflammatory mediators in the brain. Moreover, our data suggest that 1,25-VitD$_3$ supplementation alters the phenotype of T cells and increases numbers

Fig. 6 Quantification of leukocytes in the brain post-stroke. Immunohistochemistry was used to determine the numbers of **a** myeloperoxidase (MPO)$^+$ cells, **b** CD3$^+$ cells and **c** CD206$^+$ cells per right (ischemic) hemisphere in vehicle- and 1,25-dihydroxyvitamin D$_3$ (VitD$_3$)-treated animals at 24-h post-stroke. Vehicle: $n = 9$–11 per group and VitD$_3$: $n = 9$–10 per group. Student's unpaired t test. Data are presented as mean ± SEM. Arrows on representative images indicate examples of positive cells, and scale bars represent either 20 μm (**b**) or 50 μm (**a**, **c**)
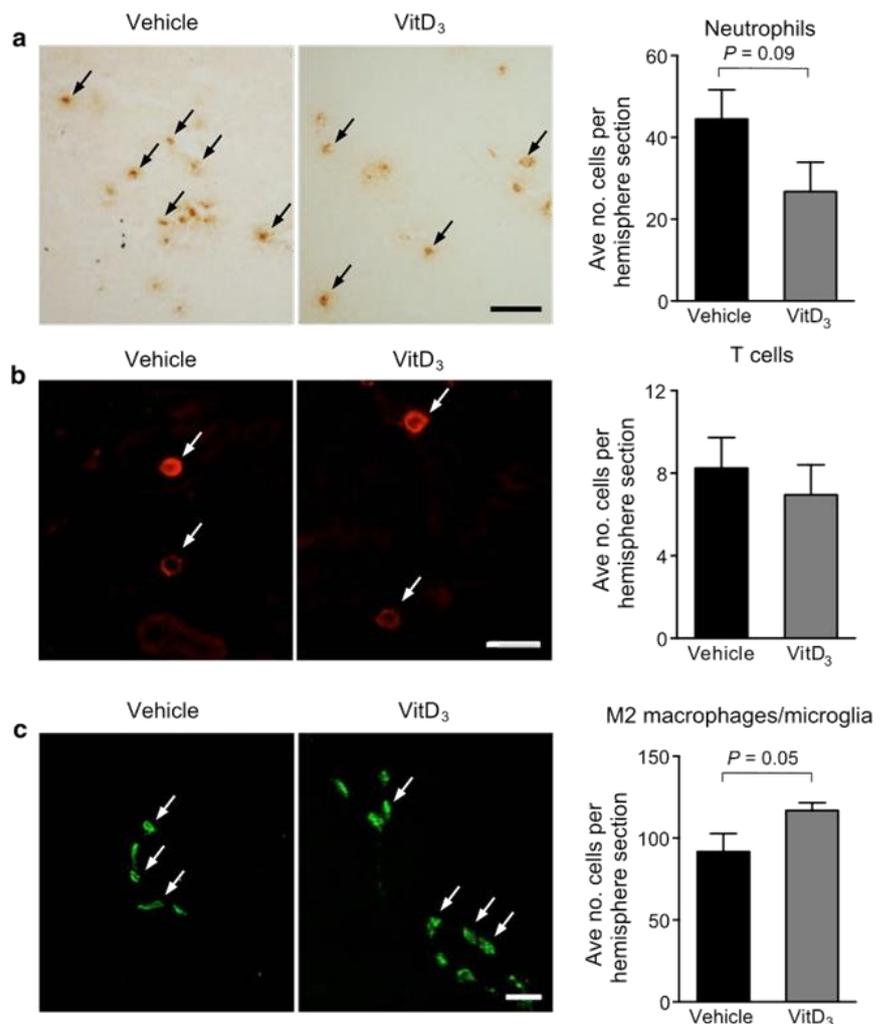
of M2 macrophages/microglia in the ischemic brain, both of which may contribute to the neuroprotection by 1,25-VitD$_3$ treatment.

Previous studies have demonstrated that the VDR and the vitamin D regulatory enzymes, 1-α-hydroxylase and 24-hydroxylase, to be expressed in non-classical tissues such as the brain and activated immune cells, suggesting that vitamin D may exert paracrine functions (Penna et al. 2007; Overbergh et al. 2000; Provvedini et al. 1983; Eyles et al. 2005). Additionally, studies have documented that the expression of the VDR and these enzymes can be altered during inflammation and disease (Luo et al. 2013; Yao et al. 2015; von Essen et al. 2010; Yang et al. 2011; Liu et al. 2006; Spanier et al. 2012). In the current study, we examined expression of the VDR (*Vdr*), 1-α-hydroxylase (*Cyp27b*) and 24-hydroxylase (*Cyp24a*), in both the brain and spleen at 24 h after stroke or sham surgery. We found that expression of the VDR was elevated in both organs after stroke. Interestingly, we observed that expression of the vitamin D-activating enzyme, 1-α-hydroxylase, was reduced in the brain after stroke, while expression of the vitamin D inactivating enzyme, 24-hydroxylase, was increased. However, in the spleen, we observed that expression of 1-α-hydroxylase and 24-hydroxylase was unchanged and undetected, respectively. These findings may imply that local levels of the active form of endogenous vitamin D may be reduced in the brain after stroke, raising the possibility that supplementation with exogenous 1,25-VitD$_3$ may be of benefit.

Indeed, we found that 1,25-VitD$_3$-supplemented animals developed a smaller infarct volume than vehicle-treated controls by 24 h. However, at this time point, there were no apparent differences in functional outcome. While 24 h is a relatively early time point for examining outcomes after stroke, we know from our previous work that infarct size is fully developed within 24 h in this model of stroke (Evans et al. 2018). Therefore, in seeking to test whether vitamin D might exert a neuroprotective effect to limit infarct development potentially by inhibiting inflammation, we chose to examine outcomes at 24 h. However, we do acknowledge the importance of evaluating the effect of 1,25-VitD$_3$ at later time points after stroke, particularly on functional recovery. Our findings are analogous to those reported by two previous studies using rat models of stroke, whereby 1,25-VitD$_3$ pre-treatment reduced infarct volume (Fu et al. 2013; Oermann et al. 2004). However, neither of these studies examined functional outcome. Moreover, the precise mechanisms by which 1,25-VitD$_3$ reduced brain injury in those studies were unclear.

To this end, we tested for evidence that 1,25-VitD$_3$ may modulate the immune response to ischemic stroke. Vitamin D can modulate the phenotype of T cells (Hart et al. 2011; Cantorna et al. 1996). For instance, in mouse models of multiple sclerosis vitamin D can down-regulate signaling pathways essential for development of Th1 and Th17 cells (Zeitelhofer et al. 2017; Mattner et al. 2000; Muthian et al. 2006; Chang et al. 2010b; Joshi et al. 2011). Moreover, vitamin D can promote the formation of Th2 and T regulatory cells (Hart et al. 2011) and limit the development of γδ T cells (Chen et al. 2005). Several studies have revealed that Th1 and γδ T cells can aggravate brain injury after stroke, and that blocking their invasion may be neuroprotective (Gu et al. 2012; Yilmaz et al. 2006; Gelderblom et al. 2012; Shichita et al. 2009). Th2 and T regulatory cells are thought to be injury-limiting in the setting of stroke (Gu et al. 2012; Liesz et al. 2009). We thus examined whether the neuroprotection by 1,25-VitD$_3$ may be associated with modulation of T cell phenotypes. In the brain, we found that neither stroke nor 1,25-VitD$_3$ had any effect on expression of Th1 or Th2 transcription factors. However, 1,25-VitD$_3$ blunted expression of the Th17/γδ T cell transcription factor, *Rorc* (ROR-γt), and enhanced expression of the T regulatory cell transcription factor, *Foxp3*. In the spleen 1,25-VitD$_3$ increased expression of *Foxp3*, but had no effect on Th1, Th2 or Th17/γδ transcription factors. Collectively, these data may indicate that 1,25-VitD$_3$ promotes the formation of T regulatory cells while inhibiting development of Th17/γδ T cells, consistent with a neuroprotective profile.

1,25-VitD$_3$ supplementation reduced mRNA expression of pro-inflammatory cytokines, *Il1β* (IL-1β), *Il6* (IL-6), *Tgfβ1* (TGF-β) and *Il23a* (IL-23a). Interestingly, these cytokines are thought to play key roles in the function of both Th17 and γδ T cells (Vantourout and Hayday 2013). Treatment with 1,25-VitD$_3$ had no effect on expression of *Il10* (IL-10), an immunosuppressive cytokine often involved in T regulatory cell function (Taylor et al. 2006); however, T regulatory cells may limit injury and excessive inflammation via other mechanisms (Sakaguchi et al. 2009). We also observed a reduction in *gp91phox* (NOX2) expression, a key producer of superoxide and mediator cellular damage following ischemic stroke (De Silva et al. 2011).

As 1,25-VitD$_3$ can reduce leukocyte recruitment to injured tissues (Pedersen et al. 2007; Korf et al. 2012; Grishkan et al. 2013), we examined its effect on leukocyte infiltration into the brain following stroke. We observed a trend for 1,25-VitD$_3$-treated animals to have fewer infiltrating neutrophils but no apparent effect on T cells. It is also possible that 1,25-VitD$_3$ reduces recruitment of other types of immune cell subsets or that it modulates their functional phenotype rather than migration to the site of post-stroke injury. It is important to note that 24 h represents a relatively early pathological time point after stroke with significant immune cell infiltration continuing after this time point (Gelderblom et al. 2009; Benakis et al. 2016). We also examined the effect of 1,25-VitD$_3$ on the numbers of M2-polarized macrophages/microglia in the brain after stroke. Studies have reported that M2 macrophages/microglia are likely to be protective in the

setting of stroke by reducing inflammation and coordinating repair processes (Benakis et al. 2014; Chu et al. 2015; Hu et al. 2012). We observed a strong trend for 1,25-VitD$_3$ to augment numbers of CD206$^+$ M2 macrophages/microglia in the brain after stroke.

As mentioned above, there is a strong rationale to gain a deeper understanding of how altered levels of vitamin D—prior and/or subsequent to stroke—might impact on the degree of ensuing brain injury. Following on from our previous finding that low baseline vitamin D levels did not impact on outcome measures at 24 h (Evans et al. 2017), here we have instead assessed the effect of increasing baseline levels achieved by five daily supraphysiological doses of vitamin D prior to stroke. Indeed, the present data suggest that supplementing mice with the active form of vitamin D prior to stroke can reduce the extent of brain injury. With regard to therapeutic relevance for acute clinical stroke, our data are important in terms of proof-of-concept. However, a limitation is that 1,25-VitD$_3$ was administered only prior to stroke induction, and clearly, studies are now required in which post-stroke treatment of 1,25-VitD$_3$ is evaluated. While our data suggest that 1,25-VitD$_3$ can also modulate the immune response to brain injury following stroke, at least part of this protection may occur via non-immune mechanisms, such as inhibiting excitotoxicity (Taniura et al. 2006; Brewer et al. 2001), stimulating production of neurotrophic factors (Neveu et al. 1994; Naveilhan et al. 1996; Landel et al. 2016) or improving blood brain barrier integrity (Won et al. 2015). It is also noteworthy that we administered 1,25-VitD$_3$ to mice that were vitamin D replete. Whether a similar or greater level of neuroprotection might be achieved in vitamin D-deficient animals by 1,25-VitD$_3$ therapy will be important to clarify.

In conclusion, these findings indicate that administration of vitamin D can attenuate infarct development following stroke possibly by modulating the inflammatory response to cerebral ischemia. Therefore, vitamin D supplementation may represent a novel direction for limiting the impact of acute stroke.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in these studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Specifically, the studies were approved by Monash University Animal Ethics Committees and performed in accordance with the National Health and Medical Research Council of Australia guidelines for the care and use of animals in research. This article does not contain any studies with human participants performed by any of the authors.

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261

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