TITLE PAGE

Title: Colchicine Therapy in Acute Coronary Syndrome Patients acts on Caspase-1 to Suppress NLRP3 Inflammasome Monocyte Activation

Robertson. Colchicine suppresses monocyte inflammasome in ACS

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ABSTRACT

Rationale: Inflammasome activation, with subsequent release of pro-inflammatory cytokines IL-1β and IL-18, has recently been implicated in atherosclerosis-associated inflammation.

Objective: To assess in acute coronary syndrome (ACS) patients (1) inflammasome activation in circulating monocytes and (2) whether short-term oral colchicine, a recognised anti-inflammatory agent that has been shown to be cardio-protective in clinical studies, might acutely suppress inflammasome-dependent inflammation.

Methods and Results: ACS patients (n=21) were randomised to oral colchicine (1 mg followed by 0.5 mg 1 hour later) or no treatment, and compared with untreated healthy controls (n=9). Peripheral venous blood was sampled pre- (day 1) and 24 hours post- (day 2) treatment. Monocytes were cultured and stimulated with ATP. Analysis of key inflammasome markers was performed by ELISA. IL-1β secretion increased by 580.4 % ($p<0.01$) in ACS patients compared to controls but only with ATP stimulation. Untreated ACS patients secreted significantly higher levels of IL-18 vs healthy controls independent of ATP stimulation ($p< 0.05$). Colchicine treatment in ACS patients markedly reduced intracellular and secreted levels of IL-1β vs pre-treatment levels ($p<0.05$ for both), as well as significantly reducing *pro-caspase-1* mRNA levels by 57.7 % and secreted caspase-1 protein levels by 30.2 % vs untreated patients ($p<0.05$ for both).

Conclusions: Monocytes from ACS patients are “primed” to secrete inflammasome-related cytokines and short-term colchicine acutely and markedly suppresses monocyte caspase-1 activity, thereby reducing monocyte secretion of IL-1β.

SUMMARY STATEMENT

Inflammasome activation in monocytes is elevated in ACS patients versus healthy subjects. Acute colchicine therapy dramatically suppresses this activation, via inhibition of caspase-1 gene transcription leading to reduced secretion of IL-1β, supporting a beneficial role for colchicine in atherosclerosis.

KEY WORDS

Atherosclerosis; Acute coronary syndromes, inflammasome, monocytes; colchicine

ABBREVIATION LIST

ASC: apoptosis-associated speck-like protein containing caspase recruitment domain
CAD: coronary artery disease
CRP: C-reactive protein
DAMPs: danger-associated molecular patterns
IL-1β: Interleukin-1β

IL-18: Interleukin-18

IL-6: Interleukin-6

NLRP3: Nucleotide-binding oligomerisation domain-like receptors, pyrin domain-containing 3

INTRODUCTION

Inflammation is central to the pathogenesis of atherosclerotic plaque progression and athero-thrombotic vascular events with systemic inflammatory markers, *e.g.* C-reactive protein, correlating strongly with prognosis in acute coronary syndrome (ACS) patients (1). Indeed, these patients are particularly vulnerable to recurrent events in the early post-ACS period, likely driven by a pan-vascular inflammatory process resulting in a higher prevalence of vulnerable non-culprit plaque (2-4). Recent studies have demonstrated that the NLRP3 (nucleotide-binding oligomerisation domain-like receptors, pyrin domain-containing 3) inflammasome, a cytosolic multiprotein complex found in monocytes, plays a key role in atherosclerosis-associated inflammation (5, 6).

The NLRP3 inflammasome is the most-widely studied and clinically relevant inflammasome and is unique in its ability to be activated by a diverse range of stimuli, of which mechanisms are still being elucidated. In the context of atherosclerosis, inflammasome activation is proposed to be two phase process – firstly, a stress signal (*e.g.* cholesterol crystals, LPS, or danger-associated molecular patterns (DAMPs)) is sensed by NLRP3 which "primes" the inflammasome complex, leading to the assembly of NLRP3, apoptosis-associated speck-like protein containing caspase recruitment domain (ASC) and pro-caspase-1 (7, 8). Activation is completed following a second signal, for example extracellular ATP leading to rapid potassium efflux, caspase-1 activation and secretion of active IL-1β and IL-18 (9-12). IL-1β, in turn, is a key inflammatory cytokine that participates in athero-thrombosis. For example, IL-1β deficient mice are characterised by reduced atherosclerotic lesions (13), while IL-1β infusions in Apo $E^{-/-}$ mice enhances aortic plaque development (14). Moreover, we have recently shown, in ACS patients, that trans-coronary IL-1β and IL-18 levels strongly correlate with disease activity (15).

Colchicine is an inexpensive potent anti-inflammatory drug. The LoDoCo trial demonstrated that long-term colchicine therapy in patients with stable coronary artery disease, lead to a reduced number of acute events over a 3 year period (16). We have recently demonstrated that short-term colchicine therapy in ACS patients reduces local coronary production of the inflammasome-specific cytokines, IL-1β and IL-18 as well as downstream IL-6 (15). Several anti-inflammatory mechanisms of colchicine have previously been reported including the inhibition of NLRP3 inflammasome protein assembly in macrophages by disrupting microtubule formation (17) as well as reducing neutrophil infiltration (18). Nonetheless, the mechanism of action of colchicine in the setting of ACS is still to be determined. Accordingly, we aimed here to characterise in *ex vivo* monocytes isolated from ACS patients (1) the

mechanisms of inflammasome activation and (2) the effects of colchicine therapy on cytokine secretion and inflammasome protein assembly.

MATERIALS AND METHODS

Patient Selection, Treatment and Sample Collection

Adult patients (> 21 years old) admitted at Royal Prince Alfred Hospital with an ACS were invited to participate in the study. ACS was defined as the clinical presentation characterised by recent onset chest pain, associated with ECG changes and/or positive cardiac enzymes - creatine kinase or troponin T, as per AHA guidelines (19). Patients were randomised in a 1:1 fashion to receive either colchicine (1 mg, followed by 0.5 mg 1 hour later) or no therapy. Peripheral vein blood samples were drawn on day 1 (before colchicine administration in the active treatment group) and on day 2 (24 hours after the first sample) (Figure 1). In the active treatment arm, colchicine was administered immediately after the first blood sample was drawn and at least 20 hours before the second blood sample. Additionally, healthy individuals with no known co-morbidities, no documented coronary artery disease or cardiovascular risk factors were sampled (only day 1 sample) and served as controls. The study protocol was approved the local Ethics Review Committee and all patients gave informed written consent before participating in this study.

Isolation and Culture Monocytes

Venous blood was collected in EDTA tubes and immediately centrifuged at 1,400 rpm for 15 minutes to remove plasma. Peripheral blood mononuclear cells were isolated using Lymphoprep (Cedarlane, Ontario, Canada) according to manufacturer's instructions and as previously described (20). Cells were re-suspended in Dulbecco's Modified Eagle Medium supplemented with 5 % fetal bovine serum and a full blood cell count was performed. Monocytes were seeded at $8x10^4$ cells/well on a 48-well plate in growth media and non-adherent cells were removed 1 hour later. Cells were cultured for 3 hours, followed by stimulation of half the cells with ATP (Sigma-Aldrich, St Louis, USA; 5 mM) for 30 minutes (Figure 1). Notably, ATP stimulation was conducted to activate the $P2X_7$ receptor leading to potassium efflux, thereby completing inflammasome activation (8). Monocyte lysates and cell media were collected and stored at -80°C for downstream analysis.

Protein Analysis

Changes in cellular or excreted protein levels were measured by enzyme-linked immunosorbent assay (ELISA), to determine the expression of key inflammasome related markers including: IL-18 (MBL International, Woburn, USA), IL-1β, IL-6 and pro-IL-1β (RnD

systems, Minneapolis, USA) and caspase-1 (Cusabio, Wuhan, China). Importantly, the antibodies used in IL-1β and pro-IL-1β ELISA are specific to each antigen. Results from cell lysates were normalised to total protein concentration as determined by a BCA assay.

RNA Isolation and RT-PCR

RNA was isolated using RNeasy Isolation Kit (Qiagen, USA) according to the manufacturer's instructions. cDNA was synthesised using Iscript (Biorad, USA) and then subjected to qRT-PCR performed using IQ Sybr mix (Biorad). Primers used for amplification are detailed below. Delta delta Ct method was used to determine gene changes (21).

	Forward (5'-3')	Reverse (5'-3')
Pro-IL-18	ATCGCTTCCTCTCGCAACAA	TCCAGGTTTTCATCATCTTGC
Pro-IL-1β	CAGAAGTACCTGAGCTCGCC	GAAGCCCTTGCTGTAGTGGT
NLRP3	CTTCTCTGATGAGGCCCAAG	GCAGCAAACTGGAAAGGAAG
Pro-caspase-1	ACTGCCCAAGTTTGAAGGACA	CACTTCCTGCCCACAGACAT
ASC (PYCARD)	CTCCTCAGTCGGCAGCCAAG	CAAGTCCTTGCAGGTCCAGT
Gapdh	TTCAACAGCGACACCCACT	TTCCTCTTGTGCTCTTGCT

Statistical Analysis

Continuous variables are reported as mean ± standard error of the mean (SEM). Differences in continuous variables (*e.g.* IL-1β concentration) were analysed via paired or unpaired t-test as appropriate, and Welch correction utilised where data sets had non-equal variances. Multiple comparisons were analysed with one-way ANOVA. Proportional differences in categorical variables were tested via the use of Fisher's Exact Test. All tests were 2-tailed with the acceptable type 1 error set at $P<0.05$. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, USA).

RESULTS

Between May and June 2014, 21 consecutive consenting ACS patients were enrolled, of whom 10 received colchicine and 11 received no therapy. Additionally, 9 healthy volunteers were also sampled (7 males and 2 females, mean age 40 years old). Baseline characteristics for the colchicine-treated and colchicine-untreated groups are shown in table 1. Groups were comparable in all categories, although there was a trend to higher rates of previous MI and revascularsation procedures (either PCI or surgery) in the no treatment arm (previous MI 11 % vs 55 %, p = 0.063). Of note, the use of recommended drugs in the setting of ACS was very high in both groups, with 100 % use of aspirin, all but one patient on a thienopyridine, more than 80 % on statins and more than 70 % on β-blockers. The size of myocardial infarct as assessed by creatinine kinase elevation was also similar between groups.

Monocytes from ACS patients secrete inflammasome-related cytokines

IL-1β

In unstimulated conditions, monocytes isolated from healthy controls and ACS patients exhibited similar intracellular levels of pro-IL-1β and IL-1β (pro-IL-1β: 37.28 ± 8.17 vs 49.16 ± 7.17 pg/µg protein; IL-1β: 116.76 ± 11.59 vs 179.58 ± 69.03 pg/µg protein, respectively). Similarly, secreted levels of IL-1β in non-stimulated cells did not differ between healthy control and ACS groups (49.69 ± 5.63 and 49.77 ± 4.43 pg/mL, respectively). ATP stimulation did not significantly change intracellular pro-IL-1β but non-significantly increased intracellular IL-1β levels in ACS patient monocytes versus healthy controls (pro-IL-1β: 126.33 ± 35.77 vs 83.97 ± 19.01 pg/µg protein; intracellular IL-1β: 382.0 ± 96.60 vs 235.40 ± 41.96 pg/µg, respectively). Notably, ATP stimulation of ACS patient monocytes also resulted in a significant increase in IL-1β secretion compared with healthy subjects (436.70 ± 95.33 pg/mL vs 123.20 ± 48.39; p = 0.008; Figure 2A). To directly compare the effects of ATP stimulation between groups, the percentage increase in pro-, intracellular and secreted IL-1β between unstimulated and ATP-stimulated monocytes was analysed. ATP-stimulation lead to comparable changes in pro-IL-1β, however markedly increased intracellular and secreted IL-1β in ACS v healthy monocytes (intracellular 314.7 ± 52.92 % v 215 ± 18.61 %, respectively, p=0.091 and secreted: 823.3 ± 157.6 % v 242.9 ± 67.84 %, respectively, p<0.01; Figure 2B).

IL-18

Intracellular (unstimulated: control 13.99 ± 1.62, ACS 39.05 ± 7.93 pg/µg protein, p = 0.006; ATP-stimulated: control 18.29 ± 3.00, ACS 74.74 ± 26.29 pg/µg protein, p = 0.045) and secreted (unstimulated: control 7.10 ± 3.35, ACS 53.26 ± 7.70 pg/mL, p<0.001; ATP-

stimulated: control 19.73 ± 10.56, ACS 73.33 ± 21.08 pg/mL p = 0.032) IL-18 levels were significantly higher in ACS patients compared to healthy controls (Figure 2C). However, in contrast to IL-1β, ATP increased monocyte IL-18 secretion from both healthy controls and ACS patients to a comparable extent in both groups (248.9 ± 57.53% and 183.5 ± 57.69%, respectively; Figure 2D).

Plasma cytokine levels

Plasma levels of IL-18 and IL-1β were similar between groups, however IL-6 levels were significantly higher in ACS compared to healthy controls (35.10 ± 8.79 vs 6.70 ± 0.86 ng/mL, respectively; p = 0.04) (Supplemental Figure 1).

Caspase-1

Secretion of the active p20 caspase-1 subunit was not significantly different between ACS patients and healthy controls, and was unaffected by ATP-stimulation (unstimulated: 192.88 ± 27.53 vs 268.60 ± 67.91 pg/mL, respectively; ATP-stimulated: 176.29 ± 24.69 vs 247.59 ± 48.91 pg/mL, respectively).

Inflammasome-related transcript levels

In unstimulated conditions, *pro-IL-18* mRNA in ACS monocytes was significantly higher compared with healthy subjects (100.30 ± 7.79 % vs 160.70 ± 13.19 %; p = 0.034), however mRNA levels of *ASC*, *NLRP3*, *pro-caspase-1* and *pro-IL-1β* were otherwise similar between groups (Figure 3). In either group, ATP stimulation did not further increase mRNA transcript levels (Supplemental Figure 2).

As ATP stimulation markedly increased intracellular and secreted IL-1β in ACS monocytes (suggesting complete inflammasome activation), colchicine or no treatment effects were expressed as a ratio of ATP-stimulated IL-1β levels /unstimulated IL-1β levels. Conversely, as pro-IL-1β, IL-18, secreted caspase-1 and transcript levels of *ASC*, *NLRP3*, *pro-caspase-1*, *pro-IL-1β* and *pro-IL-18* were not significantly increased by ATP stimulation, colchicine effects on these mediators were analysed in unstimulated conditions only.

Colchicine reduces intracellular and secreted IL-1β in monocytes from ACS patients

On day 2, monocytes from untreated ACS patients exhibited comparable levels of intracellular and secreted IL-1β versus day 1 (D1) samples (Figure 4 A-B, red bars). However, colchicine treatment significantly reduced intracellular and secreted IL-1β levels versus pre-treatment

levels (intracellular IL-1β: D1 252.40 ± 39.01 % vs D2 137.90 ± 16.72 %, p = 0.008; Figure 4A; secreted IL-1β: D1 903.60 ± 262.50 % vs D2 307.9 ± 99.28, p = 0.010; Figure 4B).
Pro-IL-1β and intracellular levels of IL-18 were unaffected by colchicine treatment (Figure 4C), however secreted IL-18 levels was reduced (colchicine-treated D1 60.41 ± 14.57 vs D2 33.67 ± 3.48, p = 0.09; Figure 4D). Peripheral venous plasma levels of IL-18 or IL-1β were not affected by colchicine treatment (Supplemental Figure 3).

Colchicine treatment reduces pro-caspase-1 mRNA levels but no other inflammasome-related mRNA transcripts

To investigate whether colchicine affected gene transcription, RNA was isolated from ACS patient monocytes and levels of key inflammasome transcripts were measured by qRT-PCR. There was no significant difference in mRNA levels for *pro-IL-1β*, *pro-IL-18*, *ASC* or *NLRP3* between ACS monocytes on day 1 and day 2, in either treated or untreated patients (Figure 5 A-D). *Pro-caspase-1* mRNA levels in non-treated ACS monocytes were significantly higher on day 2 (144.40 ± 21.50 %) versus day 1 (101.10 ± 0.36 %), p = 0.038. This rise was not observed in colchicine-treated ACS patients (D1: 102.70 ± 0.82 % vs D2: 86.71 ± 14.97 %; p = 0.275; Figure 6A). In non-treated patients, *pro-caspase-1* mRNA levels increased between day 1 and 2 by 51.94 ± 22.09 %, whereas, in colchicine treated patients, levels decreased by 5.73 ± 12.48 % (p = 0.04; Figure 6B).

Colchicine reduces caspase-1 protein secretion

Secretion of the active p20 caspase-1 subunit in monocytes from untreated patients did not significantly differ between day 1 and 2 (p = 0.38). However, in colchicine treated patients, caspase-1 secretion decreased significantly from 249.7 ± 41.03 pg/mL on day 1 to 120.0 ± 16.51 pg/mL on day 2 (p = 0.017; Figure 6C). Between sampling time points p20 caspase-1 secretion increased by 75.52 ± 39.52 % in non-treated patients, while in treated-patients secretion decreased by 30.19 ± 14.28 % (p = 0.03; Figure 6D).

DISCUSSION

In this study we have shown for the first time that (i) that monocytes from ACS patients exhibit features of inflammasome activation and (ii) that acute colchicine treatment significantly attenuates caspase-1 mRNA transcript levels and protein secretion, in turn markedly reducing cellular and secreted IL-1β levels (Figure 7).

Increased production of inflammasome-related cytokines from ACS patient monocytes

In vitro studies have previously determined that inflammasome activation is a two-step process– firstly involving a stress signal to 'prime' the inflammasome complex and a second signal (ATP) which results in complete activation, with active caspase-1 cleaving pro-IL-1β into active secreted IL-1β (8). In our study, ATP stimulation resulted in a significant increase in IL-1β secretion in ACS patients compared with healthy controls, suggesting that, in ACS patient monocytes, the inflammasome components are assembled and the complex is 'primed', yet requires a second signal (*e.g,* ATP) to complete activation, leading to IL-1β secretion. Conversely, in healthy subjects, the inflammasome is not primed and therefore IL-1β secretion is markedly less after ATP stimulation. This concept is supported by similar levels of pro-IL-1β mRNA levels at baseline between healthy controls and ACS patients. Monocytes from patients with active rheumatoid arthritis behave similarly, displaying higher sensitivity to ATP stimulation compared with healthy subjects (22). In the setting of ACS, it is possible that cholesterol crystals, present in atherosclerotic plaques and exposed after plaque rupture, induce inflammasome activation (5). However other factors such as the complement system (in particular C5a), TNFα (23), reactive oxygen species (24), and positive feedback from IL-1β or the inflammasome complex itself, which is released after cell apoptosis (25), may also contribute to inflammasome "priming". Indeed, the intense inflammatory milieu present in vulnerable atherosclerotic plaque might prime the inflammasome by several mechanisms, sustaining the inflammatory process. Accordingly, the mechanisms underlying our findings may be independent of cholesterol crystals, but dependent upon the inflammatory response secondary to plaque rupture and/or myocardial infarction. In this environment, ATP (the second stimulus required for complete inflammasome activation) might be secreted by inflammatory cells or aggregated platelets (26), alternatively it might be released from activated monocytes, leading to autocrine activation of the $P2X_7$ receptor (27). We have previously shown release of inflammasome-related cytokines in the coronary circulation in ACS patients, supporting the concept of complete inflammasome activation *in vivo* (15).

Our study also found higher IL-18 protein levels in monocytes isolated from ACS patients than those isolated from healthy controls, a pattern conserved when treated with ATP, suggesting that IL-18 release from ACS monocytes may be independent of NLRP3 inflammasome activation. These findings might be explained by the fact that IL-18 is constitutively expressed in peripheral blood mononuclear cells (PBMC) (28) and may be cleaved by either inflammasome activated caspase-1 cleavage or by other mechanisms, which may include caspase 3 (29), proteinase 3 (30), human mast cell chymase (31) or meprin beta (32). Consistent with secreted protein, *pro-IL-18* mRNA was also increased in monocytes from ACS patients compared to those from healthy subjects. In our model, ATP had no effect on *pro-caspase-1* mRNA levels nor were there differences in caspase-1 secretion, which is inconsistent with *in vitro* findings (33). This could be explained by methodological differences *e.g.* cultured *in vitro* models *versus ex vivo* preparations, absence of an experimental inflammasome priming stressor in our study *i.e.* LPS, or related to disease state, but in any case requires further study. We also demonstrated that gene levels of the inflammasome components were not significantly different between ACS and healthy subjects. These included *NLRP3*, which has previously been reported to be increased in coronary artery disease (CAD) patients and ACS patients compared to control groups (34, 35). In contrast to our study, which examined purified monocytes, these studies investigated mRNA expression in PBMCs, and therefore differences in expression may be derived from other mononuclear cells such as leucocytes. Importantly, these studies both show that NLRP3 levels altered with statin treatment, this may account for why we have not observed a change in our study. Together, our data indicate that inflammasome activation in ACS monocytes is not dependent on transcriptional regulation.

In our study, acute colchicine treatment lead to a dramatic reduction in intracellular and secreted levels of IL-1β in ATP-stimulated monocytes as well as non-significantly reducing IL-18 secretion. A corresponding decrease in pro-IL-1β mRNA was not observed, thus suggesting colchicine does not alter gene transcription of IL-1β and reduction in protein levels is due to a post-translational cleavage. Such findings are at least partially consistent with our previous study, where coronary levels of IL-1β and IL-18 were significantly reduced by colchicine therapy (15). In this study, the less marked effect on IL-18 secretion can be explained by the fact that IL-18 might also be secreted by non-monocyte resident cells in the vessel wall or other circulating leucocytes, on which colchicine could also inhibit, in contrast, the reduction in IL-1β secretion into the coronary circulation can be attributed, at least partly, to the action of colchicine on monocyte activation.

Colchicine has previously been shown to have multiple mechanisms of actions. They include inhibition of inflammasome activation by preventing crystals presentation, via attenuation of microtubule polymerisation (36). A recent study also demonstrated that colchicine impaired microtubule function, thereby blocking ASC co-localisation with NLRP3, thus preventing inflammasome complex assembly and activation (17). Here, we describe a potential additional mechanism of action, which to our knowledge has not previously been demonstrated, of reducing inflammasome activation by decreasing caspase-1 mRNA transcript levels, and therefore caspase-1 protein levels. Accordingly, when the inflammasome is completely activated by ATP, there is insufficient active caspase-1 available to cleave pro-IL-1β into its active secreted form (Figure 7). This effect appears to be specific to caspase-1 as there was no effect on the mRNA levels of other genes known participate in inflammasome assembly. The results of this study together with previous findings (17, 37) indicate that colchicine may act on multiple steps of the inflammasome pathway to prevent inflammatory cytokine release.

Limitations

Study limitations include a relatively small sample size, only investigating one inflammasome activator (ATP) and while monocytes only were investigated in our study, it is possible that colchicine might have inhibitory effects on non-monocyte inflammatory cells. Further, due to our stimulation protocol, it was required to culture monocytes for a short time, which may have impacted on their phenotype; this time however was minimised to less than 4 hours and is similar to previously published methods using *ex vivo* monocytes (20, 27, 38, 39). Importantly, this method was used consistently across all samples, allowing for relative comparisons to be made between patients and/or treatments. Our study was designed to test the effects of short-term colchicine administration on monocyte inflammasome activation; long-term use of this drug and effects on clinical outcome or any side effects were not evaluated. Moreover, we have not elucidated how colchicine interacts with monocytes and the intracellular messaging cascade that ultimately leads to inhibition of *pro-caspase-1* mRNA synthesis. Lastly, pro-caspase-1 gene knockout studies would be required to confirm effects on mRNA synthesis.

CONCLUSIONS

Our results suggest, for the first time, that (1) the NLRP3 inflammasome in monocytes from ACS patients is primed to secrete key athero-inflammatory cytokines and (2) that acute colchicine administration markedly inhibits monocyte secretion by IL-1β, at least in part by reducing caspase-1 mRNA levels and protein secretion. Together, these data indicate a novel, clinically relevant, anti-inflammatory mechanism of action for colchicine in unstable

atherosclerotic disease. Our findings, therefore, provide further insights into colchicine's athero-protective properties that have been previously demonstrated by us and others, and support its therapeutic role in atherosclerosis-associated inflammation.

CLINICAL PERSPECTIVE

Inflammation plays a pivotal role in atherosclerotic plaque progression and instability, leading to clinical events. Notably, inflammasome activation, with its downstream cytokines, IL-1β and IL-18 have been implicated in atherosclerosis-associated inflammation, and therefore may be targets for inhibition. We demonstrate here that short-term colchicine therapy in patients presenting with an acute coronary syndrome significantly reduces caspase-1 transcript and protein levels in monocytes. This, in turn, leads to reduced production of IL-1β, a pro-inflammatory cytokine that is associated with plaque instability and recurrent coronary events.

DECLARATIONS OF INTEREST

None Declared

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AUTHOR CONTRIBUTION

S.R. was responsible for drafting and revising the manuscript. S.R., G.J.M., J.Y.B. and C.A.P. all played an important role in the design of the study, recruitment of subjects, experimental procedures and data analysis. D.S.C. and C.B. provided further technical support with regard to data analysis. S.P. was responsible for concept design and provided final approval of the manuscript.

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TABLES

Table 1. Baseline characteristics of ACS patients, randomised to colchicine or no treatment

	Colchicine (n=9)	No Colchicine (n=11)	P value
Age (y) Mean (SD)	69.80 (12.53)	67.55 (14.57)	0.86
Female (%)	2 (22)	3 (27)	1
Diabetes mellitus (%)	2 (22)	4 (36)	0.64
Hypertension (%)	6 (67)	10 (91)	0.15
Dyslipidaemia (%)	7 (78)	8 (73)	1
Family history (%)	3 (33)	5 (46)	0.66
Current smoker (%)	4 (44)	3 (27)	0.66
Previous MI (%)	1 (11)	6 (55)	0.06
Previous PCI (%)	1 (11)	3 (27)	0.59
Previous CABG (%)	1 (11)	3 (27)	0.59
Renal impairment (%)	1 (11)	3 (27)	0.59
Medications			
Aspirin (%)	9 (100)	11 (100)	-
Thienopyridines (%)	8 (89)	11 (100)	0.48
β-blockers (%)	7 (78)	8 (73)	1
Nitrates (%)	1 (11)	4 (36)	0.31
ACE-I / ARA-2 (%)	4 (44)	7 (64)	0.39
Ca-blockers (%)	1 (11)	1 (9)	1
Diuretics (%)	2 (20)	5 (46)	0.36
Statins (%)	9 (100)	9 (82)	0.48
Hypoglycemics (%)	2 (22)	4 (36)	0.64
Insulin (%)	1 (11)	2 (18)	1
Fibrates (%)	1 (10)	1 (9)	1
Blood tests (Mean (SD))			
Haemoglobin (g/L)	134.40 (19.46)	127.72 (24.16)	0.47
White cell count (x10^9/L)	8.84 (2.12)	8.46 (2.24)	0.65
Platelet count (x10^9/L)	234.70 (63.05)	239.91 (77.03)	0.76
Creatinine (μmol/L)	88.50 (34.77)	92.36 (41.15)	0.43
Cholesterol (mmol/L)	3.25 (0.65)	4.88 (1.95)	0.14
Glucose (mmol/L)	7.12 (1.27)	8.45 (3.44)	0.76
Max CK (U/L)	649.87 (582.03)	695.63 (569.63)	1
Max Trop (ng/L)	1037.00 (1024.76)	2016.38 (2913.15)	1

SD: standard deviation; ACE-I: angiotensin converting enzyme inhibitors; ARA-2; angiotensin II receptor antagonist; CK: creatinine kinase

FIGURES

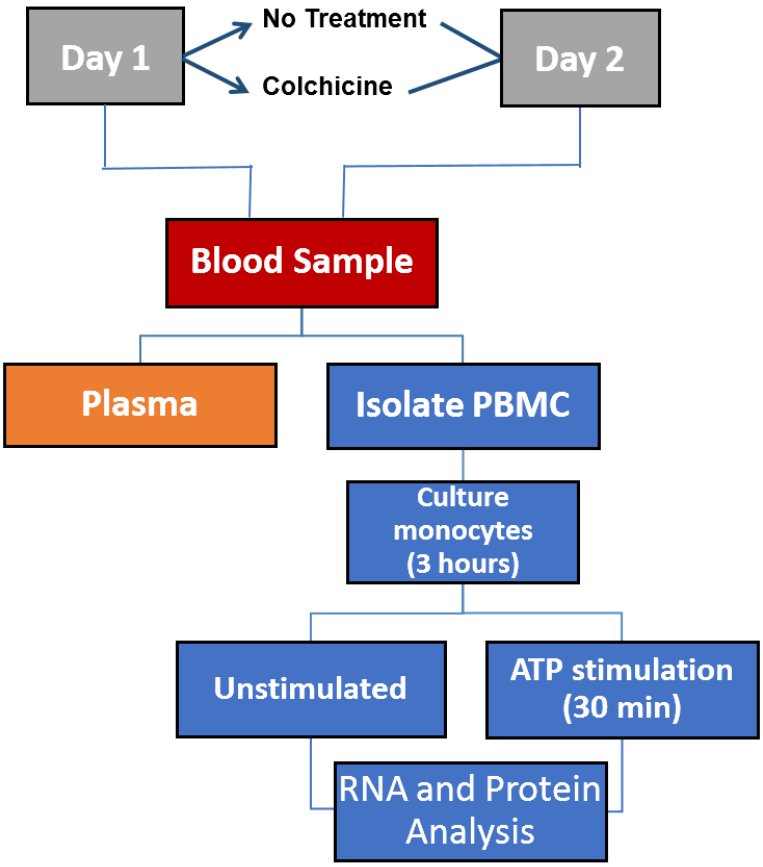

Figure 1. Sampling and *ex vivo* stimulation protocol.

PBMC; peripheral blood mononuclear cells.

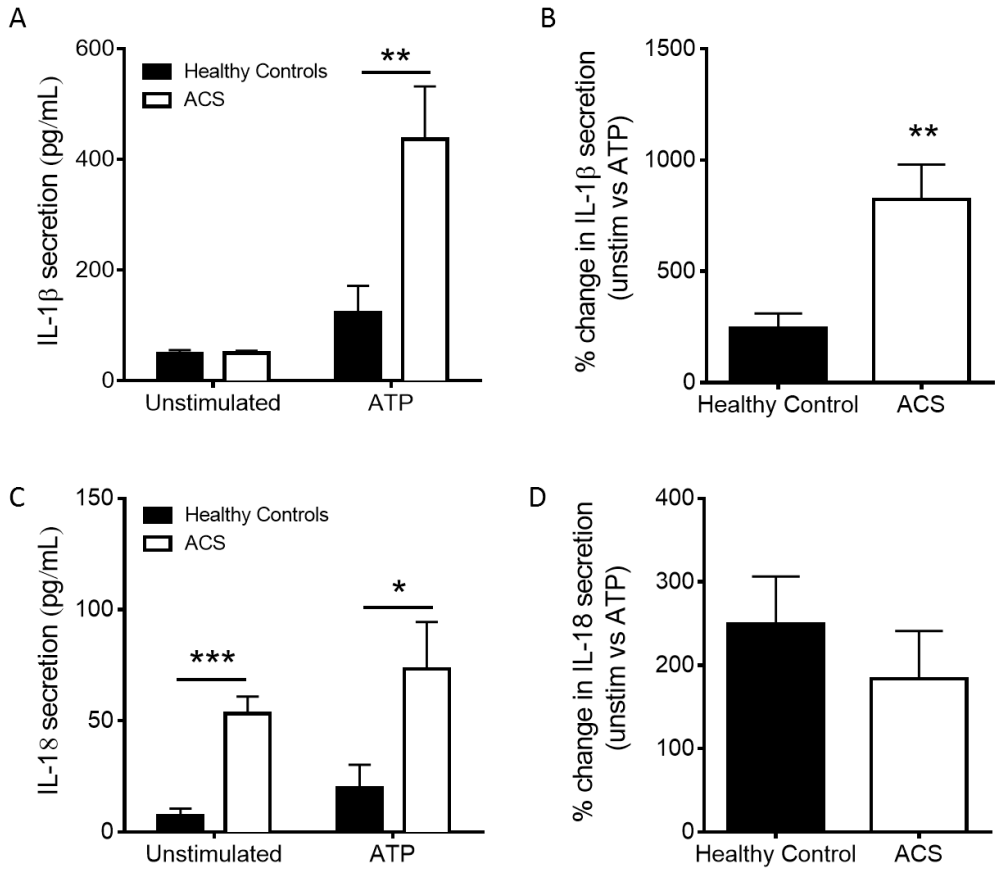

Figure 2. Inflammasome markers are increased in monocytes of ACS patients compared to healthy controls.

Protein levels of key inflammasome-specific cytokines IL-1β (A) and IL-18 (C) were measured using ELISA in culture media of monocytes isolated from either healthy controls (n = 9, black bars) or untreated acute coronary syndrome patients (ACS; n = 11, white bars). B and D indicate the percentage change in secretion of cytokines in ATP-stimulated monocytes compared to patient matched unstimulated monocytes. Results are presented as mean ± SEM; * $p<0.05$ $p<0.01$ and $p<0.001$.

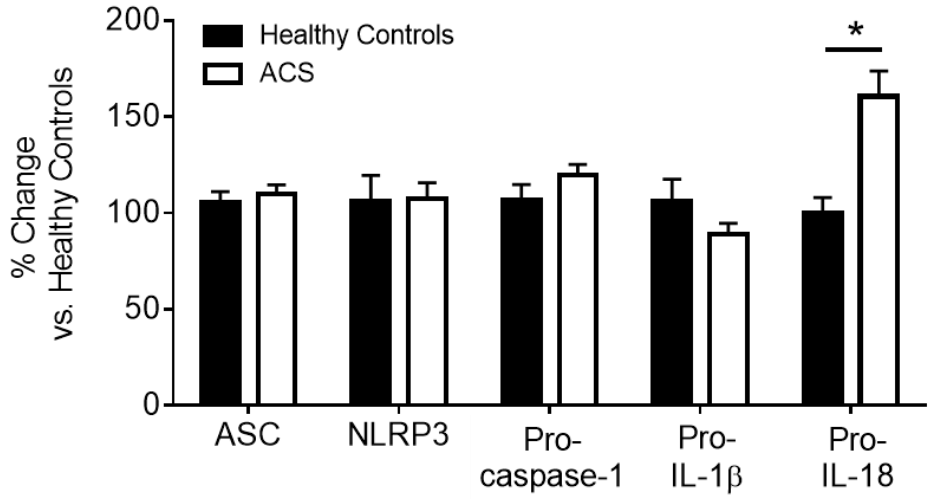

Figure 3. Transcript levels of inflammasome mRNAs in monocytes.

Expression analysis of inflammasome-related mRNA transcripts from RNA isolated from monocytes. *ASC* (apoptosis-associated speck-like protein containing a CARD); *NLRP3* (NOD-like receptor family, pyrin domain containing 3). Results normalised to *GAPDH* expression and expressed as percentage mean ± SEM of healthy control; * $p<0.05$.

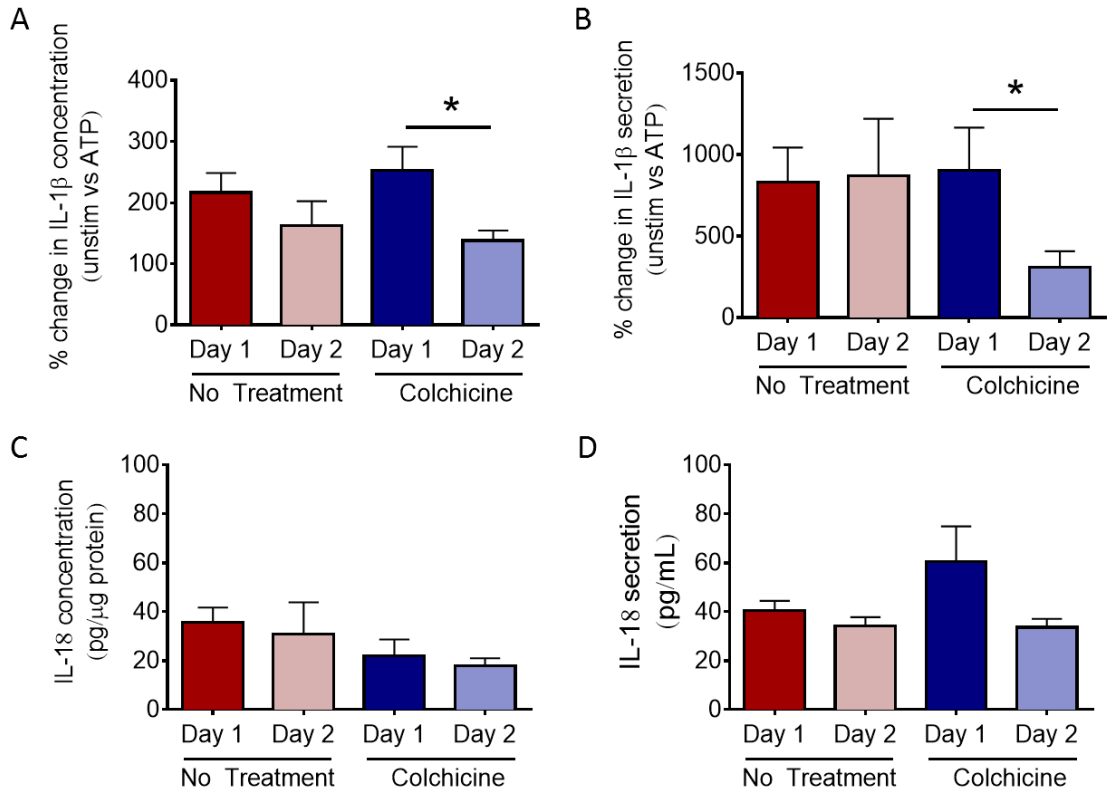

Figure 4. Colchicine reduces IL-1β secretion from monocytes of ACS patients.

IL-1β (A and B) and IL-18 (C and D) were measured using protein specific ELISA in lysates (A and C) or in culture media (B and D) of monocytes isolated from acute coronary syndrome (ACS) patients at baseline (Day 1; red or blue) and ACS patients 24 hours later (Day 2) following either no treatment (n = 11, light red) or oral colchicine treatment (n = 9, light blue). For IL-1β expression levels from ATP-stimulated cells are expressed as a percentage of patient-matched non-stimulated cells; data are presented as mean ± SEM; * $p<0.05$.

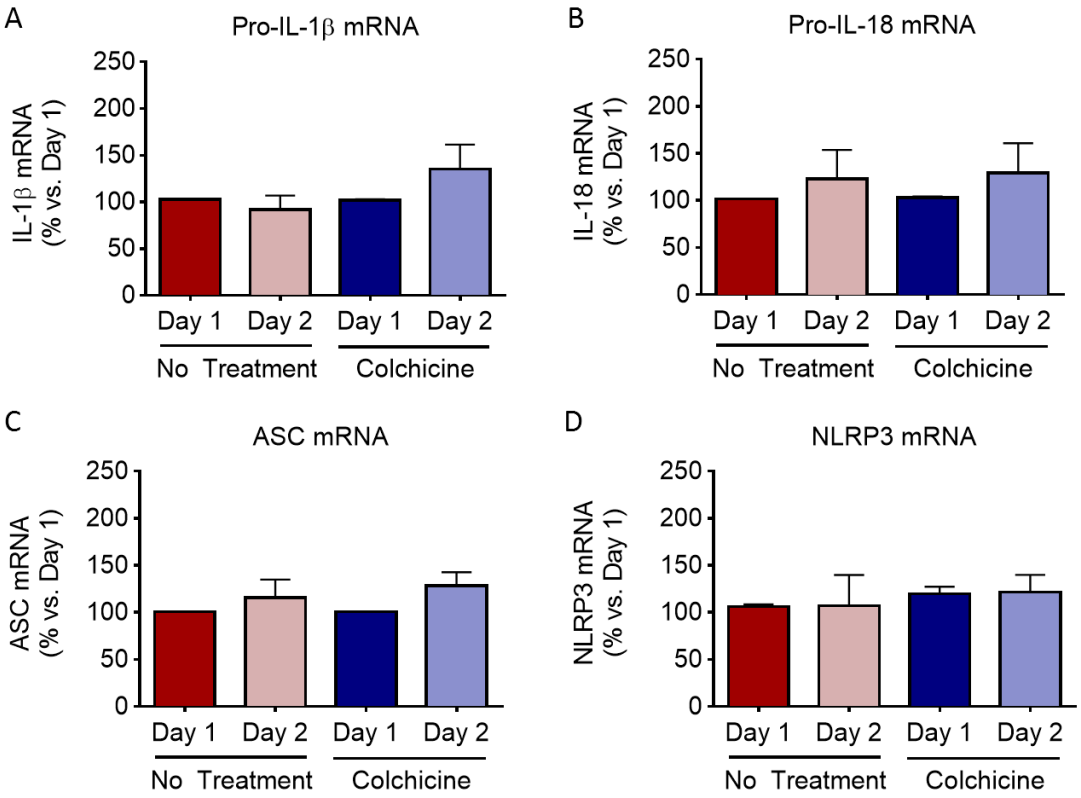

Figure 5. Colchicine suppression of the inflammasome does not alter transcription of inflammasome transcript.

Expression analysis of inflammasome-related mRNA transcripts from RNA isolated from monocytes from either acute coronary syndrome patients (ACS) at baseline (Day 1; red or blue) and ACS patients 24 hours later (Day 2) following either no treatment (n = 11, light red) or oral colchicine treatment (n = 9, light blue). Genes measured: *pro-IL-1β* (A); *pro-IL-18* (B); *ASC* (apoptosis-associated speck-like protein containing a CARD) (C); and *NLRP3* (NOD-like receptor family, pyrin domain containing 3) (D). Results are presented as mean ± SEM.

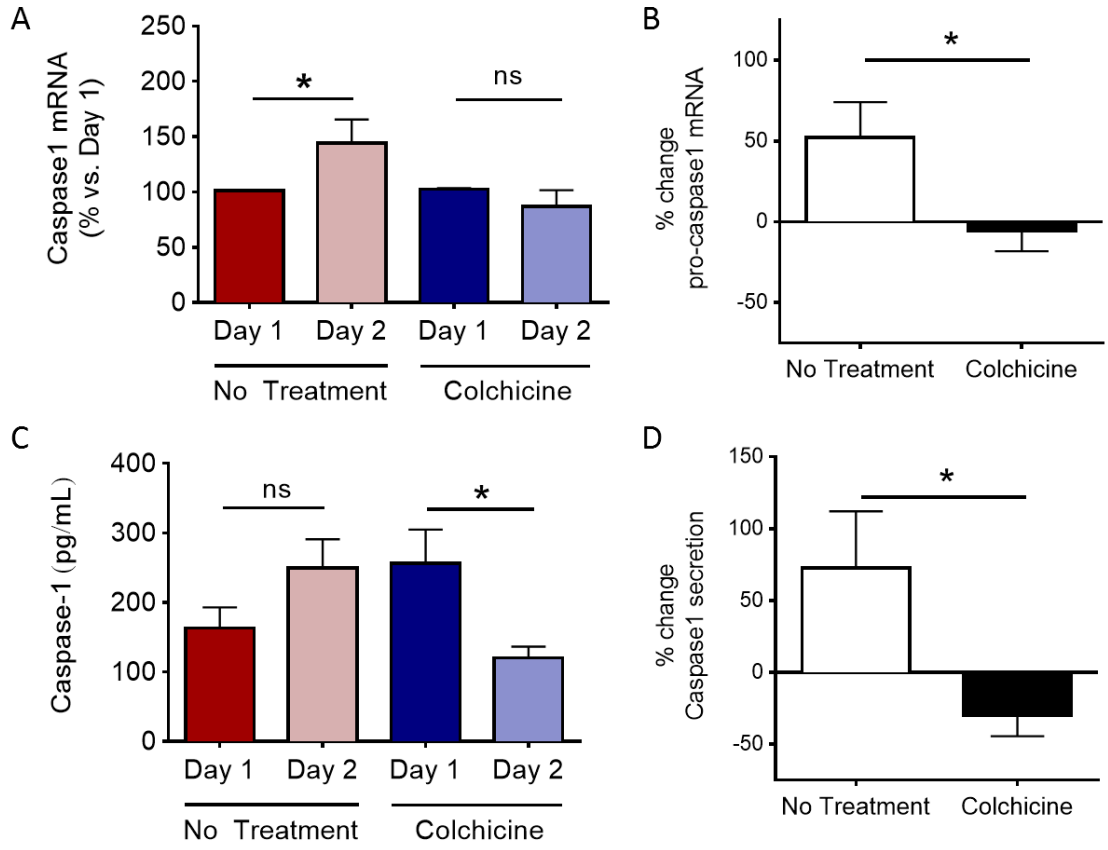

Figure 6. Colchicine significantly reduces monocyte transcript levels of caspase-1 and secretion of active caspase-1.

Caspase mRNA transcript (A) or secreted caspase-1 protein (C) levels from monocytes isolated from acute coronary syndrome patients at baseline (Day 1; red or blue) and 24 hours later (Day 2) following either no treatment (n = 11, light red) or oral colchicine treatment (n = 9, light blue). B and D illustrate the percentage change in matched samples from Day 1 to Day 2. Results are presented as mean ± SEM; ns: non-significant; * $p<0.05$.

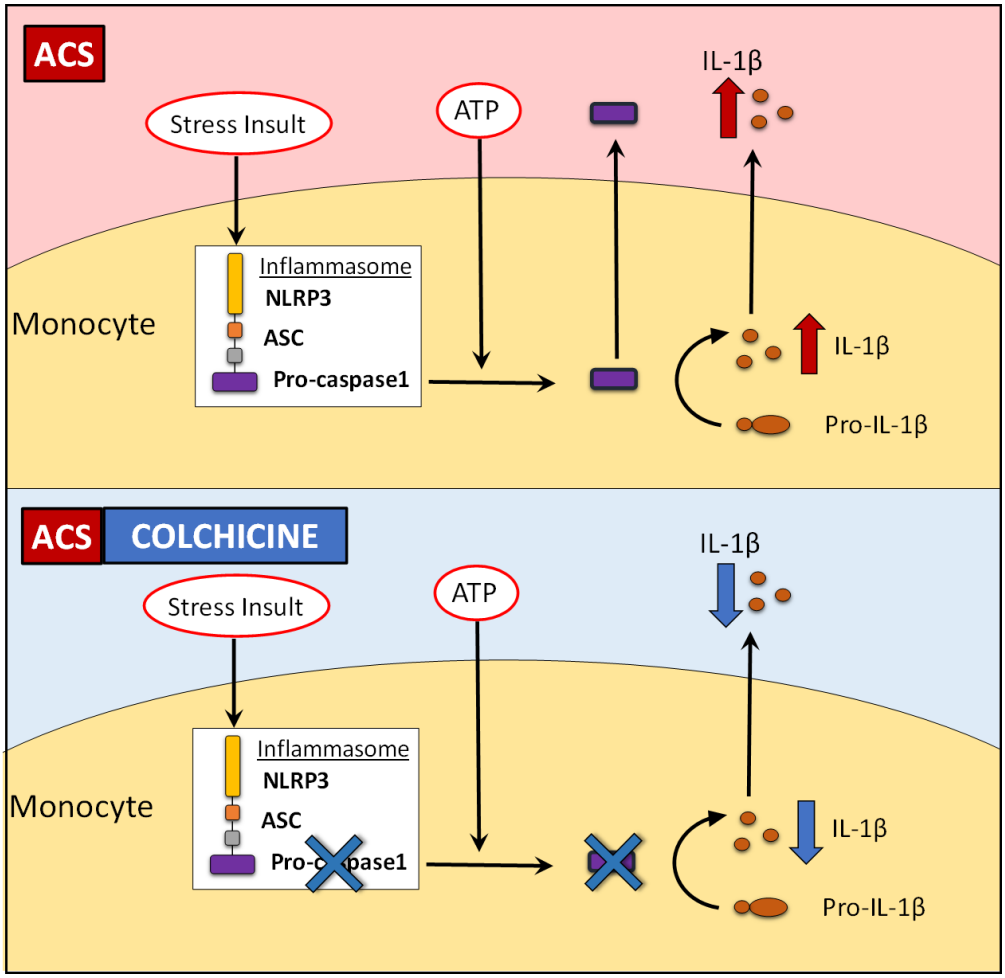

Figure 7. Effect of short-term colchicine on caspase-1 expression and IL-1β production in monocytes from acute coronary syndrome patients.

The NLRP3 inflammasome in monocytes from ACS patients is primed, and can readily produce and secrete IL-1β following final stimulation with ATP (top panel). With short-term colchicine therapy, *pro-caspase-1* mRNA synthesis and secreted caspase-1 protein levels are significantly inhibited. This, in turn, arrests pro-IL-1β cleavage, with subsequent reduced secretion of active IL-1β, despite ATP stimulation (bottom panel).