Neutrophils in patients with chronic coronary syndrome exhibit delayed spontaneous apoptosis and resistance to regulatory T cell-induced apoptosis – Brief Report

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Short title: Delayed neutrophil apoptosis in coronary patients

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Abstract

Background: Persistent inflammation is linked to poor outcomes in patients with chronic coronary syndrome (CCS). The inflammatory state in atherosclerotic disease has been associated with activation of neutrophils as well as with regulatory T cell (T_{reg}) deficiency. The role of T_{regs} in the regulation of neutrophil survival has been postulated recently. Here, we investigated neutrophil apoptosis along with the potential impact of T_{regs} on neutrophil apoptosis in patients with CCS, compared to healthy controls.

Methods: Twenty patients with CCS and 19 healthy controls were included. Neutrophil apoptosis was assessed after 5h culture with or without interleukin(IL-)10, TNF or LPS. Neutrophil phenotype was evaluated through flow cytometry analysis of surface receptors (CD66b and CXCR4) and *ex vivo* release of cytokines and granule proteins. The ability of T_{regs} to induce neutrophil apoptosis was examined in autologous neutrophil-T_{reg} co-cultures.

Results: Spontaneous neutrophil apoptosis was significantly delayed in patients compared to controls (10.3% vs. 19.2%, p=0.025). Also, neutrophils from patients overexpressed CD66b and CXCR4 and were more prone to release proinflammatory mediators. Notably, T_{regs} induced neutrophil apoptosis in healthy subjects, but not in patients, indicating a loss of T_{reg}-mediated regulation in the latter. There was no evidence that IL-10 had any influence on neutrophil apoptosis. However, cell-to-cell contact was found essential for T_{reg}-induced neutrophil apoptosis.

Conclusions: Patients with CCS display delayed neutrophil apoptosis and a pro-inflammatory neutrophil phenotype that is resistant to T_{reg}-mediated apoptosis.

Neutrophil dysfunction may contribute to persistent inflammation in patients with CCS, and as such constitute a novel therapeutic target.

Key words:

Coronary artery disease, Chronic coronary syndrome, Inflammation, Apoptosis, Neutrophils, Regulatory T cells

Introduction

The relationship between inflammation and atherosclerosis is well established, as shown in experimental, epidemiological and clinical studies [1]. Despite optimal management of classical risk factors like hyperlipidemia and hypertension, systemic low-grade inflammation promotes recurrent cardiovascular events in patients with chronic coronary syndrome (CCS), a condition former known as stable coronary artery disease [2].

Growing evidence implicates neutrophils as pivotal mediators in atherosclerotic inflammation [3]. Epidemiological studies consistently link elevated neutrophil counts to incident atherosclerotic cardiovascular disease and mortality [4]. Increased neutrophil activation is also a well-known feature of the acute coronary syndrome (ACS) [5-7], while the occurrence of neutrophil activation in patients with CCS has been less studied. However, elevated neutrophil counts as well as neutrophil granule proteins, including myeloperoxidase (MPO), matrix metalloprotease (MMP)-8 and MMP-9, have been reported in patients with CCS, indicating a heightened neutrophil activation status [8-11]. A few previous studies have also investigated whether neutrophils from patients with CCS are more prone to activation *ex vivo* compared to neutrophils from healthy controls, although with inconsistent results [12-14].

Neutrophils have a very short life, around 6 h, in circulating blood, They are inherently programmed to migrate back to the bone marrow via CXCR4 expression and die by constitutive apoptosis [15]. Neutrophil apoptosis is considered to be a critical mechanism for resolving acute inflammation. A delay in the apoptotic process has been associated with increased neutrophil effector functions [16,17]. Accordingly, delayed neutrophil apoptosis has been observed in a number of chronic inflammatory disorders [18-20]. A few previous studies have also described delayed neutrophil apoptosis in patients with coronary artery disease, though with focus on ACS [21,22].

Whether a delay in neutrophil apoptosis occurs in the stabilized phase after an ACS has not been clarified.

In patients with CCS, reduced numbers as well as impaired function of regulatory T cells (T_{regs}) have been a consistent finding [23-25]. T_{regs} are primarily recognized for their ability to modulate adaptive immunity. However, it has also been demonstrated that murine as well as human T_{regs} are able to promote neutrophil apoptosis and inhibit neutrophil activity [26,27]. Interestingly, the role of dysregulated neutrophil-T_{reg} interaction in chronic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus, has been proposed [28,29]. Whether a dysregulated neutrophil-T_{reg} interaction takes place in patients with CCS has not previously been explored.

In the present study, we hypothesized that delayed neutrophil apoptosis is a feature of the persistent inflammatory state in patients with CCS. We examined neutrophil apoptosis, along with neutrophils´ activation/aging status and their susceptibility to autologous T_{regs}, in patients with CCS and healthy control subjects.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. A detailed, expanded method sectionis is available in the Data Supplement.

<u>Study population</u>

Twenty patients with CCS were recruited; all had angiographically verified coronary artery disease and a prior history of ACS. Patients were not eligible if they were more

than 75 years of age, suffered from severe heart failure, immunological disorders, neoplastic disease, received treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin), or had a history of acute or recent (<2 months) infection, major trauma/surgery or any revascularization procedure. For the control group (n = 19), volunteers with generally equal age and sex distribution were randomly selected from the Swedish Population Register. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki, and the protocol was approved by the Ethical Review Board of Linkoping. Written informed consent was obtained from all study participants.

Cell isolation and culture

Venous blood was collected in the morning in a fasting state. $CD4^{+}CD25^{+}CD127^{-/low}$ T_{regs} were isolated from peripheral blood mononuclear cells (PBMCs) using an EasySep Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit, and CD4+ T cells were isolated from PBMCs using a CD4+ T Cell Isolation Kit human. Neutrophils were isolated from whole blood using Polymorphprep.

For immediate staining, neutrophils were incubated with mouse anti-human CXCR4 APC and mouse anti-human CD66b PE antibodies for 15 min (Table S1). They were analysed within 1h using a Gallios Flow Cytometer.

Pure neutrophils, or co-cultures with neutrophils and T_{regs} or $CD4^{+}$ T cells were seeded in 96-well plates at concentrations of 0.5 $x10^{6}$ cells/ml for 2.5 h or 5 h, with or without interleukin (IL)-10, tumor necrosis factor (TNF) or lipopolysaccharide from *E. coli* (LPS).

Neutrophil apoptosis and activation

Neutrophils were incubated for either 2.5 h or 5 h in different series of experiments. After 2.5 h, supernatants were stored at -80°C until further analysis. After 5 h, cells were stained with mouse anti-human CD66b PE, anti-human Annexin V BV42 and mouse anti-human CD4 BV510 antibodies (if the sample contained T_{regs} or $CD4^+$ cells) in Annexin V Binding Buffer, followed by staining with SYTOX Red dead cell stain. Cells were analysed within 1 h using a Gallios Flow Cytometer to monitor apoptosis and activation status of neutrophils. Gating strategies are shown in Figure S1.

Transwell experiments

Isolated neutrophils and T_{regs} were seeded in a 48-well cell culture plate with or without Millicell Cell Culture Inserts.

Proteins in plasma and supernatants

Tumor necrosis factor (TNF), interferon(IFN)-γ, interleukin(IL)-6, IL-1β and IL-18 were measured in plasma via Olink Target 48, with an inter-assay CV of 7% and an intra-assay CV of 6%. Matrix metalloproteinase(MMP)-9, MMP-8, myeloperoxidase (MPO) in plasma were measured using a Luminex Multiplex Assay, with an intra-assay CV of 3.2%. MMP-9, MMP-8, MPO, TNF, IFN-γ, IL-6, IL-10, IL-1β and IL-18 were also measured in supernatants using a Luminex Multiplex Assay, with an inter-assay CV of 16.6% and an intra-assay CV of 8.1%. Measuements were performed by the Affinity Proteomics-Unit of SciLifeLab, Stockholm, Sweden.

Statistics

All statistics were performed using the software IBM SPSS Statistics version 29.0.2.0. For comparisons between groups, a Mann-Whitney U test was performed. For comparison of paired data from the same individual, a Wilcoxon signed rank test

was performed. For analysis of concentration-dependency, the Greenhouse-Geisser Test was performed.

Results

Cohort characteristics

All patients with CCS were clinically and metabolically stable with no symptoms of angina. There were no differences between patients and controls regarding age (median age 65 *vs* 64 years), sex distribution (females 50 % *vs* 53 %), body mass index or smoking status. Also, the protein levels in plasma were comparable between groups, except for higher levels of TNF and IL-10, as well as slightly but not significantly higher levels of IL-6, in the CCS group. Blood samples were collected from patients at a median of 11.5 months (interquartile range: 5.8-13 months) after an ACS. Cohort characteristics are presented in Table S2.

Neutrophil apoptosis is delayed in patients with CCS

The apoptotic rate of neutrophils was assessed after 5 h culture in medium only (spontaneous apoptosis) or in the presence of either anti-inflammatory (IL-10) or pro-inflammatory (TNF, LPS) stimuli. As shown in Figure 1A, the spontaneous apoptosis was significantly reduced in CCS patients compared to healthy controls (10.3% vs. 19.2%, $p = 0.025$). A similar result was seen after 5 h treatment with IL-10 (10.1% vs. 19.8%, $p = 0.020$). Under inflammatory conditions (TNF or LPS), the differences in neutrophil apoptosis between patients and controls did not reach statistical significance.

Neutrophils exhibit an aged and pro-inflammatory phenotype in patients with CCS

The surface expression of CD66b was used as a marker of neutrophil activation [30]. After 5 h in medium only or in the presence of IL-10, TNF, or LPS the expression of CD66b was significantly higher in patients than in controls (Figure 1B).

The surface expression of CD66b and CXCR4, the latter representing neutrophil aging [15], was also assessed immediately after cell isolation. Neutrophils from CCS patients exhibited increased surface expression of CD66b as well as increased expression of CXCR4, compared to neutrophils from healthy subjects (Figure 1C-D).

To further investigate the neutrophil activation status, we measured the release of neutrophil granule proteins (MMP-9, MMP-8, MPO) and cytokines (TNF, IFN-γ, IL-6, IL-18, IL-1β) after a shorter period of incubation (2.5 h) with or without IL-10, TNF or LPS. There were no differences between patients and controls when neutrophils were cultured in medium only. However, upon LPS stimulation, the neutrophils from patients secreted higher levels of MMP-9, MMP-8, TNF, IFN-γ, IL-6 and IL-1β compared to controls. Also, the neutrophils from patients released higher levels of MMP-9 upon TNF treatment (Figure 1E).

<u>T_{regs} induce neutrophil apoptosis in healthy controls, but not in patients with CCS</u>

We then proceeded to examine the effect of autologous T_{regs} on neutrophil apoptosis by co-culturing the cells at varying ratios (neutrophils:T_{regs} 1:0, 10:1, 4:1 and 1:1) for 5 h with or without IL-10, TNF or LPS. In the controls, T_{regs} induced neutrophil apoptosis in a concentration-dependent manner under all conditions, whereas no such effect was observed in the patients (Figure 2A-B). When T_{reg}-induced neutrophil apoptosis was compared between patients and controls, the controls exhibited a significantly higher apoptotic rate at 5 h (with or without IL-10) at all ratios (Figures S2A-B). Autologous $CD4^+$ T cells did not induce neutrophil apoptosis, neither in

healthy controls nor in CCS patients (Figure S2A-D for between-groups analysis, Figure S3 for within-group analysis).

T_{reg} survival and IL-10 secretion in neutrophil-T_{reg} co-cultures do not differ between patients with CCS and healthy controls

To determine whether differences in T_{reg} survival contributed to the discrepancy in T_{reg}-induced neutrophil apoptosis between groups, we assessed the T_{reg} viability under various conditions. Neither T_{reg} nor $CD4^+$ T cell survival differed between patients and controls under any conditions (Figure S4A-B).

To gain an insight into the functionality of T_{regs}, IL-10 was measured in co-culture supernatants of patients and controls. In controls, the concentrations of IL-10 appeared to increase at ratios 4:1 and 1:1 (Figure S4C-D). However, the absolute levels were low (0.2 - 1.1 pg/ml), and no significant differences were observed between patients and controls.

T_{reg}-induced neutrophil apoptosis is not dependent on IL-10, but rather on cell-to-cell contact

Next, we investigated two possible mechanisms of T_{reg}-induced neutrophil apoptosis. To determine whether IL-10 was a potential mediator of T_{reg}-induced neutrophil apoptosis, neutrophils from healthy volunteers were treated with increasing concentrations of IL-10 (1 pg/ml, 1 ng/ml, 100 ng/ml) for 5 h, mimicking concentrations in co-culture supernatants (mentioned above) as well as concentrations used in previous *in vitro* studies [31,32]. None of the IL-10 concentrations showed any effect on the apoptotic rate (Figure 2D). To further assess whether T_{regs} used cell-to-cell contact to regulate neutrophil apoptosis, transwell experiments were performed to physically separate T_{regs} and neutrophils via a membrane (mesh) at

ratio 4:1. As shown in Figure 2E, the separation completely abrogated T_{reg}-induced neutrophil apoptosis.

Discussion

The main finding of the present study is that spontaneous neutrophil apoptosis, a key process in the resolution of inflammation, was significantly delayed in patients with CCS compared to healthy controls. Concomitantly, the neutrophils in patients exhibited an aged and proinflammatory phenotype as well as a resistance to T_{reg}-induced neutrophil apoptosis.

Delayed neutrophil apoptosis has been described in several chronic inflammatory diseases [18-20]. Only a few earlier studies have investigated neutrophil death in coronary artery disease, with emphasis on ACS, a condition characterized by acute inflammation. In 2004, Garlich *et al.* [21] demonstrated that neutrophil death, including both apoptosis and necrosis, was delayed in patients with ACS (n=30), compared to patients with stable angina (n=15) and controls (n=15) after 24 h culture. Some years later, Biasucci *et al.* [22] measured spontaneous neutrophil apoptosis in patients with ACS (n=30), patients with stable angina (n=13) and controls (n=34). They found delayed neutrophil apoptosis in ACS patients after 4 h culture (10.1%), whereas the apoptotic rate in stable angina patients (28.5%) did not differ from that in controls (41.8%). In the present study, we detected a delay in neutrophil apoptosis already after 5 h in patients with CCS compared to controls. Comparisons with earlier study groups should however be drawn with caution, e.g. the apoptotic rates reported by Biasucci *et al.* [22] after 4 h culture were remarkably higher than what we found, which might indicate methodological differences. Also, we included patients who had

survived ACS, while Biasucci *et al.* [22] included stable angina patients without prior ACS. Despite being asymptomatic and without overt heart failure, it cannot be excluded that our patient cohort had a more severe, complicated coronary artery disease, hence a more proinflammatory neutrophil phenotype.

It is well established that patients with ACS exhibit a more active neutrophil phenotype, either within culprit coronary lesions or circulating in peripheral blood [3]. Whether the active neutrophil phenotype is maintained in those stabilized after a prior ACS has been less clear. Results from *ex vivo* functional assays are sparse; some have reported that circulating neutrophils in patients with CCS are primed, i.e. more prone to stimulation *ex vivo*, as compared to neutrophils in healthy controls [12,13], while others have not been able to detect any differences [14,33]. In the present study, delayed spontaneous neutrophil apoptosis in patients with CCS was clearly accompanied by increased expression of CD66b, a sensitive marker of neutrophil activation in human samples [30]. Also, the finding that patients´neutrophils were more prone to secrete cytokines and granule proteins upon stimulation *ex vivo* supported their state of heightened activity. Furthermore, the increased expression of CXCR4 indicate an accumulation of aged neutrophils in the circulation of patients with CCS. Aged neutrophils have been shown to exhibit a distinct, highly reactive phenotype, both in experimental models and in chronic inflammatory disease [34,35].

Statins are known to exert pleiotropic anti-inflammatory effects, one of which is the suppression of neutrophil activation [36,37]. Furthermore, two single-center randomized trials have shown that statins promote neutrophil apoptosis in acute inflammatory conditions [38,39]. Despite extensive treatment with statins, CCS patients in our study exhibited an increase in proinflammatory neutrophils with delayed apoptosis. These

findings indicate that statin-mediated immunomodulatory effects on innate immunity, while robust *in vitro*, may be less effective or relevant in chronic inflammation.

T_{regs} are well known for their role in controling the adaptive immune response [29]. Emerging evidence indicates that T_{regs} also have a regulatory role in the innate immune response [29]. Of note, a deficit in circulating T_{regs} is a characteristic finding in patients with both acute and chronic manifestations of coronary artery disease [23-25], as well as in patients with other chronic inflammatory diseases [40-42]. Earlier experimental studies, mainly performed on murine cells, have shown that LPS-activated T_{regs} are able to inhibit neutrophil function and promote neutrophil apoptosis [26,27]. In the present study, T_{regs} promoted neutrophil apoptosis both with and without LPS in healthy controls, while this phenomenon did not occur in patients with CCS. The reason for resistance to T_{reg}-induced neutrophil apoptosis may be multifactorial, depending on anti-apoptotic features of pro-inflammatory neutrophils [43] but also on functional defects of T_{regs}. As previously described by our group, the capacity of T_{regs} to regulate proliferation and secretion of IFN-γ and IL-10 in autologous T responder cells is impaired in patients with CCS compared to healthy controls [25]. Several studies have also shown that a chronic inflammatory environment may lead to impaired T_{reg} function depending on molecular mechanisms, such as perturbed *Foxp3* expression and epigenetic modifications [44].

IL-10 has been described as a mediator of anti-inflammatory effects exerted by T_{regs} [45,46]. In the present study, we used a wide range of concentrations of IL-10 in both neutrophil cultures and neutrophil-T_{reg} co-cultures, but were not able to demonstrate any effect on neutrophil apoptosis after 5 h. This is in agreement with earlier studies reporting that IL-10 does not affect spontaneous neutrophil apoptosis, neither after short (6 h) nor long (20 h) incubation [47,48]. However, these earlier studies have also

reported that, under *in vitro* conditions mimicking severe sepsis, IL-10 is capable of counteracting LPS-induced delay of neutrophil apoptosis [31,47,48]. Moreover, LPS-stimulated T_{regs} have been shown to induce IL-10 production in apoptotic neutrophils after 5 h co-culture, while LPS alone or unstimulated T_{regs} do not [49]. In the present study, we detected very low concentrations of IL-10 in supernatants after 5 h co-culture, both with and without LPS, speaking against a significant role of IL-10 in this experimental model for T_{reg}-induced neutrophil apoptosis. On the other hand, our findings clearly show that T_{reg}-induced neutrophil apoptosis was dependent on cell-to-cell contact. The results are in agreement with previous studies reporting that human T_{regs} exert suppressive effects through cell-to-cell contact [49,50].

Several limitations of our study should be acknowledged. The neutrophil-T_{reg} co-cultures were performed at cell ratios that do not reflect physiological conditions in peripheral blood where the neutrophil:T_{regs} ratio is normally 100:1. However, the *in vitro* approach using ratios from 1:1 to 10:1 is in line with previous mechanistic studies [26,27,51]. Another major limitation is that we did not measure the phenotype of isolated T_{regs}, which limits the understanding of why neutrophil-T_{reg} interaction was impaired in patients compared to controls. Lastly, the sample size was small and our findings should be considered preliminary until validated in a larger cohort.

Nevertheless, a strength of the study is the *ex vivo* investigation of peripheral neutrophils in human disease, addressing a critical gap in translational cardiovascular research. While numerous epidemiological studies have shown that high neutrophil counts is a causal risk factor for cardiovascular disease [4], mechanistic insights have been mainly derived from animal models [3].

To conclude, delayed spontaneous neutrophil apoptosis, along with a more active neutrophil phenotype showing resistance to T_{reg}-induced apoptosis, indicate that

neutrophils may be a major contributor to the non-resolved inflammatory state in patients with CCS. Of note, neutrophil dysfunction was observed in clinically stable patients who received optimal medical therapy according to current guidelines. Although the findings need to be confirmed in larger samples, and also explored in further mechanistic studies, they allow us to speculate about neutrophil apoptosis as a future target of therapy in patients with CCS.

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Disclosures

None.

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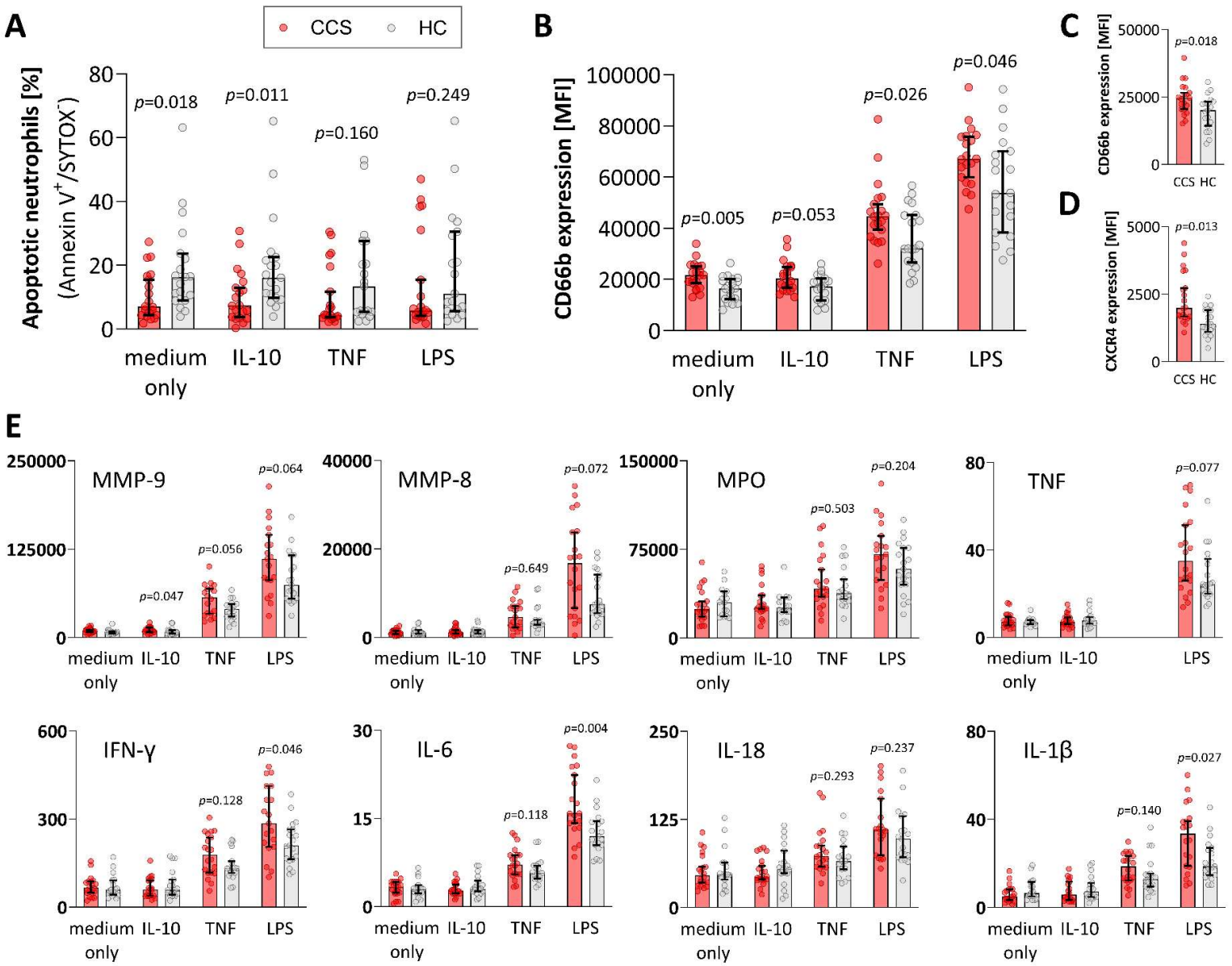
A
CCS
HC
Apoptotic neutrophils [%]
(Annexin V+/SYTOX-)
p=0.018
p=0.011
p=0.160
p=0.249
medium only
IL-10
TNF
LPS
B
CD66b expression [MFI]
p=0.005
p=0.053
p=0.026
p=0.046
C
CD66b expression [MFI]
p=0.018
D
CXCR4 expression [MFI]
p=0.013
E
MMP-9
p=0.047
p=0.056
p=0.064
MMP-8
p=0.649
p=0.072
MPO
p=0.503
p=0.204
TNF
p=0.077
IFN-γ
p=0.128
p=0.046
IL-6
p=0.118
p=0.004
IL-18
p=0.293
p=0.237
IL-1β
p=0.140
p=0.027

Figure 1: Neutrophil apoptosis, cell surface marker expression and neutrophil activation in patients with chronic coronary syndrome and healthy controls.

Isolated neutrophils from 20 patients with chronic coronary syndrome (CCS) and 19 healthy controls (HC) were cultured in medium only or medium with interleukin (IL)-10 (20 ng/ml), tumor necrosis factor (TNF, 1 ng/ml) or lipopolysaccharide (LPS, 100 ng/ml). After 5 h, flow cytometry was used to assess neutrophil apoptosis; data are presented as the percentage of apoptotic (Annexin^{+}/SYTOX^{-}) neutrophils (**A**). Flow cytometry was also used to assess the expression of CD66b; data are presented as mean fluorescence intensity (MFI) values (**B**).

Furthermore, the neutrophil expression of CD66b and CXCR4 was assessed by flow cytometry immediately after cell isolation in the same study cohort. Data are presented as MFI values of CD66b (**C**) and MFI values of CXCR4 (**D**).

Additionally, protein concentrations were measured in the same study cohort after 2.5 h of incubation in medium with or without IL-10 (20 ng/ml), TNF (1 ng/ml) or LPS (100 ng/ml) (**E**). Concentrations of matrix metalloprotease (MMP)-9, MMP-8 and myeloperoxidase (MPO), TNF, interferon (IFN)γ, IL-6, IL-18 and IL-1β are presented as pg/ml. Significances between CCS and HC in normal medium and IL-10 were all $p > 0.05$, except for MMP-9 measurement in IL-10 treated sample.

Significances are indicated between CCS and HC and all values are given as median ± 95% confidence interval.

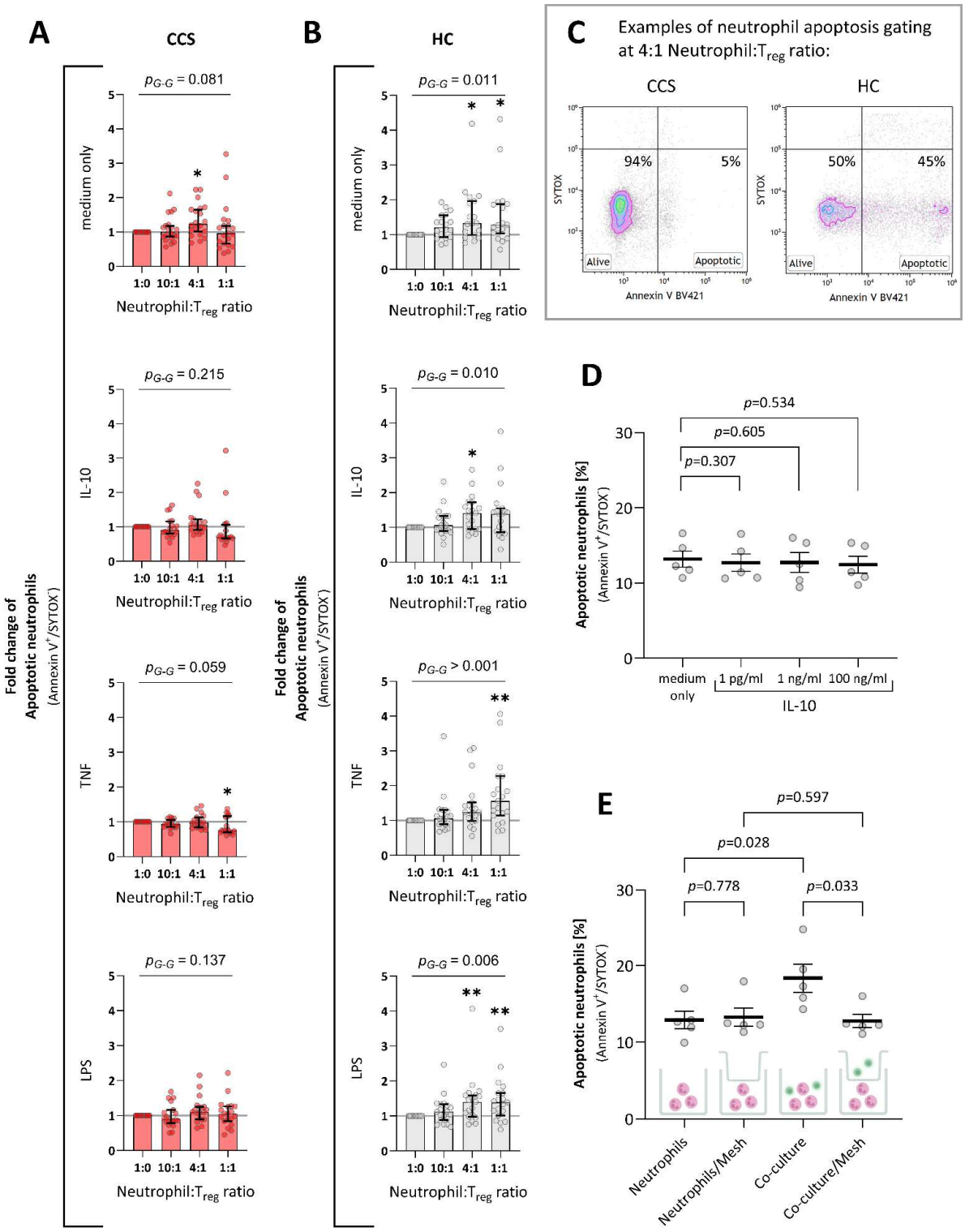

Figure 2: The effect of neutrophil-T_{reg} co-cultures on neutrophil apoptosis in patients with chronic coronary syndrome and healthy controls.

Neutrophils and T_{regs} were isolated from 20 patients with chronic coronary syndrome (CCS) and 19 healthy controls (HC). The cells were co-cultured at different ratios (neutrophils: T_{regs} 1:0, 10:1, 4:1 and 1:1) in medium only or medium with interleukin (IL)-10 (20 ng/ml), tumor necrosis factor (TNF, 1 ng/ml) or lipopolysaccharide (LPS, 100 ng/ml). After 5 h of co-culture, neutrophil apoptosis (Annexin$^+$/SYTOX$^-$) was determined by flow cytometry. The percentages of apoptotic neutrophils are presented as fold change compared to pure neutrophil culture in patients with CCS (**A**) and HC (**B**). Significances are compared to 1:0 culture within CCS and HC groups, respectively, *p*-values are adjusted for multiple comparison via Dunn; CCS (**A**): *p=0.015 in medium only ratio 4:1, *p=0.036 in TNF ratio 1:1; HC (**B**): *p=0.014 in medium only ratio 4:1, *p=0.030 in medium only ratio 1:1, *p=0.025 in IL-10 ratio 4:1, **p=0.002 in TNF ratio 1:1, **p=0.009 in LPS ratio 4:1, **p=0.006 in LPS ratio 1:1; and significance for trend is given as *p*-value of a Greenhouse-Geisser test. All values are given as median ± 95% confidence interval. Representative examples for the gating of apoptotic neutrophils as Annexin V$^+$/SYTOX$^-$ cells are shown in **C**, the full gating strategy can be reviewed in Figure S1.

Additionally, isolated neutrophils from healthy volunteers (n=5, < 50 years of age) were treated with different doses of IL-10 (1 pg/ml, 1ng/ml and 100ng/ml). Flow cytometry was used to assess neutrophil apoptosis after 5 h; data are presented as the percentage of apoptotic (Annexin$^+$/SYTOX$^-$) neutrophils (**D**). In another series of experiments, using cells from the same volunteers described in (**D**), neutrophils alone or neutrophil-T_{reg} co-cultures at ratio 4:1 were seeded +/- mesh (as graphically indicated) in medium only for 5 h, followed by flow cytometry to assess neutrophil apoptosis (**E**). *p*-values were adjusted for multiple comparison via Tukey. All values are given as mean ± SEM.