Persistent accumulation of interferon-gammaproducing CD8(+)CD56(+) T cells in blood from patients with coronary artery disease

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Abstract

Objective

There is emerging evidence for CD8⁺ T cell alterations in blood from patients with coronary artery disease (CAD). We examined whether the distribution and phenotype of CD8⁺CD56⁺ T cells differed according to the clinical manifestation of CAD.

Methods

Patients with acute coronary syndrome (ACS, n=30), stable angina (SA, n=34) and controls (n=36) were included. Blood was collected before and up to 12 months after referral for coronary investigation. CD8⁺CD56⁺ T cells were assessed by flow cytometry for expression of surface markers, apoptosis, and intracellular expression of cytokines.

Results

The proportions of CD8⁺CD56⁺ T cells were significantly higher in both ACS and SA patients compared with controls, and remained so after 3 and 12 months. This was independent of age, sex, systemic inflammation and cytomegalovirus seropositivity.

CD8⁺CD56⁺ T cells differed from CD8⁺CD56⁻ T cells in terms of lower CD28 expression and fewer apoptotic cells. Both CD8⁺ T cell subsets were positive for interferon (IFN)-γ and tumor necrosis factor, although IFN-γ was significantly more confined to the CD8⁺CD56⁺ T cells.

Conclusion

The persistent accumulation of CD8⁺CD56⁺ T cells in ACS and SA patients share several features with immunological aging. It also contributes to a larger IFN- γ ⁺ pool in blood, and may thereby hypothetically drive the atherosclerotic process in a less favorable direction.

Keywords

Acute coronary syndrome; Coronary artery disease; Cytokines; Immune system; Leukocytes

Persistent accumulation of interferon-γ-producing CD8⁺CD56⁺ T cells in blood from patients with coronary artery disease

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Introduction

Chronic immune stimulation plays an important role in atherogenesis¹. Activated T cells are prominent components of human atherosclerotic lesions²⁻³ and also, blood samples from patients with coronary artery disease (CAD), in particular acute coronary syndromes (ACS), show signs of T cell activation⁴⁻⁶. While much attention has been focused on the role of CD4⁺ T cells, the CD8⁺ T cell compartment has been less investigated. However, in human lesions, CD8⁺ T cells are almost as frequent as CD4⁺ T cells, thus not reflecting the CD4:CD8 ratio of about 2:1 normally seen in blood⁷. In addition, it was recently shown that human plaques contain more activated CD8⁺ T cells than activated CD4⁺ T cells³. A few earlier studies measuring the numbers of activated CD8⁺ T cells in the circulation of patients with CAD have shown contradictory results⁸⁻⁹.

A subpopulation of circulating T cells, the majority being CD8⁺ T cells, expresses the natural killer cell marker CD56. In infants, the numbers of circulating CD8⁺CD56⁺ T cells are extremely low, but then increase with age¹⁰. The induction of CD56 has been shown to correlate with the loss of CD28, a hallmark of immunological aging¹⁰⁻¹¹, and furthermore, the conversion to a senescent T cell phenotype is accentuated by a common pathogen, namely cytomegalovirus (CMV)¹². In addition, increased proportions of circulating CD8⁺CD56⁺ T cells are described in chronic autoimmune diseases like rheumatoid arthritis, sarcoidosis and Behcet's uveitis^{11, 13-15}. The expression of CD56 on CD8⁺ T cells has also been associated with disease activity^{11, 16}. Still, the clinical relevance of accumulated CD8⁺CD56⁺ T cells during aging and disease is far from clarified. CD56⁺ T cells are often described as cytolytic effector cells¹⁷ but conversely, CD56 has emerged as a potential marker for CD8⁺ suppressor T cells with anti-inflammatory potential¹⁸⁻¹⁹. *In vitro*, CD56⁺ T cells have shown the capacity to produce both type 1 and type 2 cytokines^{10, 15-16, 20-21}.

An increase in CD56⁺ T cells was recently reported in patients with acute myocardial infarction compared with healthy controls, independent of CMV seropositivity²². In an earlier cross-sectional study, we found that patients with stable CAD exhibited an increase in CD8⁺ T cells lacking CD28, a phenomenon that was not only determined by CMV seropositivity but also by CAD *per se*²³. In the present study, we hypothesized that the distribution and phenotype of CD8⁺CD56⁺ T cells in CAD patients would reflect disease activity, thereby changing over time. The proportions and phenotypic characteristics of CD8⁺CD56⁺ T cells were therefore longitudinally investigated in ACS and SA patients before and up to 12 months after referral for coronary investigation. In order to further characterize the functional status of these cells, the cytokine profile was examined after stimulation *ex vivo*.

Methods

Subjects

The study population consisted of 30 patients with ACS, 34 patients with SA, and 36 healthy control subjects. Coronary angiograms were performed in all patients at day 1. Blood samples were always collected prior to coronary angiography, and in ACS patients within 24 h from admission. The patients were longitudinally followed, and after 3 months samples were collected in 16 ACS and 28 SA patients, and after 12 months in 15 ACS and 28 SA patients. The ACS patients were included if they had a diagnosis of unstable angina/non-ST elevation myocardial infarction, with the diagnosis based on typical ECG-changes (ST-T segment depression and/or T-wave inversion) and/or elevated troponins. The SA patients included were referred for elective coronary angiography due to effort angina class II or III in

accordance with Canadian Cardiovascular Society Classification and without any worsening of symptoms the latest 3 months. Patients were excluded if they had severe heart failure, immunologic disorders, neoplastic disease, evidence of acute or recent (<2 months) infection, recent major trauma, surgery or revascularization procedure, treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin). The age and sex matched control subjects, randomly selected from a population based register representing the hospital recruitment area, were anamnestically healthy and received no medication. The study was conducted in accordance with the ethical guidelines of Declaration of Helsinki, and the research protocol was approved by the Ethical Review Board of Linköping University. Written informed consent was obtained from all study participants.

Monoclonal antibodies and in vivo lymphocyte profiles

Lymphocyte subpopulations from peripheral blood were analyzed by 6- or 7-color combinations. Cells were stained with Annexin V-PE or the following monoclonal antibodies: CD3-FITC (clone SK7), CD3-PerCP (clone SK7), CD4-APC (clone SK3), CD4-PE-Cy7 (clone SK3), CD8-APC-H7 (clone SK1), CD16/CD56-PE (clone B73.1/NCAM16.2), CD25-FITC (clone 2A3), CD28-PE (clone L293), CD45-PerCP (clone 2D1), CD56-APC (clone NCAM16.2), CD56-HorizonV450 (clone B159), CD56-PE-Cy7 (clone NCAM16.2), CD69-PE (clone L78), CD69-PE-Cy7 (clone L78), CD94-APC (clone HP-3D9), HLA-DR-FITC (clone L243), IFN-γ-PE (clone 25723.11), IL-10-PE (clone JES3-9D7), IL-13-PE (clone JES10-5A2), IL-17A-PE (clone SCPL1362), TNF-α-PE (clone 6401.1111), all from BD Biosciences, San José, CA, US.

As previously described²⁴, whole blood and antibodies were incubated for 15 minutes at room temperature (RT), thereafter erythrocytes were lysed with FACS™ Lysing Solution (BD Biosciences) for 15 minutes at RT. Samples were analyzed on a FACSCanto II (BD Biosciences) equipped with 3 lasers, a blue 488 nm, a red 633 nm and a violet 405 nm.

Analysis of samples was stopped when 10 000 cells were collected in the lymphocyte gate.

Data were analyzed and subpopulations gated with FACSDiva™ 6.1.2 software (BD Biosciences).

Whole blood stimulation and detection of intracellular cytokines

Whole blood stimulation was performed in a subgroup of the study population, *i.e.* 9 ACS, 14 SA and 11 control subjects. For detection of intracellular cytokines, the protocol FastImmune (BD Biosciences) for whole blood stimulation was followed. In brief, heparinized whole blood was stimulated *ex vivo* for 6h at 37°C and 5% CO₂ with anti-CD28/CD49d (BD Biosciences), 50ng/ml phorbol 12-myristate 13-acetate (PMA; SIGMA, Saint Louis, MO, US), and 1µg/ml ionomycin (SIGMA) in presence of 10µg/ml brefeldin A (BD Biosciences). Samples were then incubated 15 minutes at RT with EDTA-solution (BD Biosciences), erythrocytes were lysed and cells fixed for 10 minutes in RT with FACSTM Lysing Solution (BD Biosciences). The samples with stimulated cells were then immediately stored at -70°C. After thawing, cells were permeabilized for 10 minutes at RT with Permeabilizing solution 2 (BD Biosciences). They were washed and subsequently stained for IFN-γ, TNF, interleukin (IL)-10, IL-13 or IL-17A, followed by staining of surface markers, including early activation marker CD69, for 30 minutes at RT. After washing, cells were resuspended in 1% paraformaldehyde/phosphate buffered saline and analyzed on FACSCanto II and FACSDivaTM 6.1.2 software.

Assays for C-reactive protein, IL-6, IL-15 and cytomegalovirus seropositivity

C-reactive protein (CRP) was measured in serum using a highly sensitive latex-enhanced turbidimetric immunoassay (Roche Diagnostics GmbH, Vienna, Austria) with a detection limit of 0.03 mg/l. IL-6 and IL-15 concentrations were measured in plasma using a commercial chemiluminescent enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (QuantiGlo Chemiluminescent ELISAs, R&D systems, Abingdon, UK). The sensitivity limits of quantification for IL-6 and IL-15 was 0.48 pg/ml and 1.03 pg/ml, respectively. Anti-CMV IgG was detected in plasma samples by chemiluminescent microparticle immunoassay (CMIA) (Architect, Abbott Laboratories, Abbott Park, IL, USA). Antibody levels ≥ 6.0 AU/ml was considered positive.

Statistical analyses

IBM SPSS Statistics 19 was used for statistical analyses. Differences between ACS, SA and controls were analyzed with Kruskal-Wallis test and Mann-Whitney U-test was used for between-group analyses. Chi-square test was used for nominal data. Differences within groups were analyzed using Friedman test and if P < 0.1, Wilcoxon signed ranks test was used for pair-wise comparisons. For correlation analyses Spearman's rank correlation method was used. Multiple linear regression analysis was performed to assess the independent contribution of different factors to T cell changes. P < 0.05 was considered to be statistically significant. Values are presented as median (inter-quartile range). P-values > 0.10 are shown as nonsignificant (NS) in tables.

Results

The characteristics of patients and controls are listed in Table 1. At day 1, 11 (32%) SA patients and 19 (63%) ACS patients underwent percutaneous coronary intervention while 16 (47 %) SA patients and 5 (17 %) ACS patients were referred for coronary bypass surgery. Seven SA patients and 6 ACS patients did not undergo any vascular revascularization procedure. All patients were on low-dose aspirin and various combinations of β -blockers, calcium-antagonists and nitrates. Compared with ACS patients, SA patients were to a larger extent long-term treated, i.e. > 2 months, with statins at day 1. However, at 3 months followup, 87 % of the SA patients and 94 % of the ACS patients received statin resulting in similar LDL cholesterol levels in both groups. The levels of CRP, IL-6 and IL-15 during the 12 month period of follow-up are given in Table 2. Both SA and ACS patients exhibited higher levels of IL-6 at day 1. The levels declined significantly in both patient groups. At 12 months, the IL-6 levels in SA patients still remained at a significantly higher level compared with controls. The levels of CRP and IL-15 did not differ between groups at any time point except for higher levels in ACS patients at day 1. During follow-up, IL-6 levels in patients were significantly correlated with smoking (r = 0.32, p < 0.01), waist circumference (r = 0.38, p < 0.01) and hypertension (r = 0.28, p < 0.05). Follow-up CRP levels only showed correlations with waist circumference (r = 0.28, p < 0.05) while IL-15 lacked correlations with clinical variables. The prevalence of CMV seropositivity was similar in patients and controls.

The total numbers of leukocytes were significantly higher in both patient groups at day 1 (Table 3) and did not change during follow-up. The leukocyte differential counts in ACS and SA patients were left-shifted with increased percentages of granulocytes compared with

controls, 59 (53-63), 59 (51-64) and 54 (49-58) %, respectively, p = 0.022. Conversely, the percentages of lymphocytes were decreased in ACS and SA patients compared with controls, 30 (24-35), 29 (26-36) and 34 (31-42) %, respectively, p = 0.018, whereas the percentages of monocytes were similar, 7.9 (7.1-9.1), 7.8 (6.8-9.7) and 7.8 (6.6-8.5) %, respectively. As shown in Table 3, the total numbers of lymphocytes were higher in ACS patients compared with SA patients and controls whereas the number of CD8⁺ T cells did not differ between the groups at day 1. The numbers of lymphocytes or CD8⁺ T cells did not change in any of the patient groups during follow-up. In both ACS and SA patients, the numbers and proportions of CD8⁺CD56⁺ T cells were significantly higher compared with controls. Also, the ratios between CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells were significantly higher in ACS and SA patients compared with controls, 0.43 (0.21-0.55), 0.40 (0.23-0.66) and 0.26 (0.15-0.39), respectively, p = 0.033. At 3 and 12 months, the proportions of CD8⁺CD56⁺ T cells remained increased in both ACS and SA patients (Figure 1 a and b). The numbers of CD4⁺ T cells were higher in ACS patients, and tended to be higher in SA patients (P = 0.054), compared with controls. During follow-up, the numbers of CD4⁺ T cells in ACS and SA patients did not change. The numbers and proportions of CD4⁺CD56⁺ T cells were low in all groups.

The numbers or proportions of CD8⁺CD56⁺ T cells did not show any correlations with clinical variables such as age, sex, waist circumference or smoking, neither were there any correlations with hypertension, diabetes, prior cardiac event or number of diseased coronary vessels. Patients who were on long-term treatment with statin at day 1 (n = 37) displayed similar proportions of CD8⁺CD56⁺ T cells as did patients without statin (n = 27), 29 (19-39) % and 28 (15-35) %, respectively. There were no correlations between proportions of CD8⁺CD56⁺ T cells and levels of CRP, IL-6 or IL-15 at any time point. In patients (ACS + SA), the number of CD8⁺CD56⁺ T cells correlated with the number of CD4⁺ T cells, r = 0.55,

P < 0.001, at all time points whereas no such correlation was seen in controls. There was a correlation between proportions of CD8⁺CD56⁺ T cells and CMV seropositivity in the whole study population, r = 0.26, P = 0.011. In a multiple regression analysis identifying CMV seropositivity and CAD as independent predictors of CD8⁺CD56⁺ T cells, both CMV seropositivity and CAD remained associated with increased proportions of CD8⁺CD56⁺ T cells (β 0.22, P = 0.025 and β 0.24, P = 0.016, respectively).

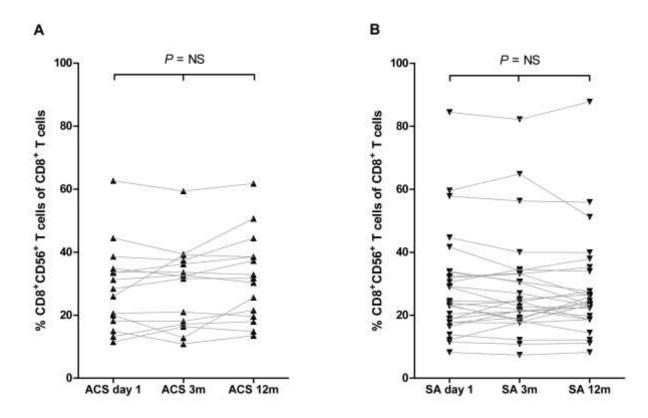


Figure 1. Proportions of CD8⁺CD56⁺ T cells in (**A**) ACS patients and (**B**) SA patients. No change in the CD8⁺CD56⁺ T cell proportions was seen neither in ACS nor in SA patients over 12 months, as assessed by Friedman test.

Lack of CD28 was more frequent in CD8⁺CD56⁺ T cells compared with CD8⁺CD56⁻ T cells; 71 (46-85) % vs. 33 (19-57) %, (P < 0.001 for the whole study population). As shown in Table 4, the proportions of CD8⁺CD28⁻CD56⁺ and CD8⁺CD28⁻CD56⁻ T cells were similar in patients and controls. The proportion of Annexin V⁺ cells was significantly lower among CD8⁺CD56⁺ T cells than among CD8⁺CD56⁻ T cells; 2.1 (0.7-4.0) % vs. 4.2 (2.7-6.5) %, (P < 0.001 for the whole study population). Moreover, the proportions of apoptotic CD8⁺CD56⁺ T

cells were lower in ACS and SA patients compared with controls while the proportions of apoptotic CD8 $^+$ CD56 $^-$ T cells were similar in the 3 groups (Table 3). The expression of CD69 was more frequent in CD8 $^+$ CD56 $^+$ T cells than in CD8 $^+$ CD56 $^-$ T cells; 5.0 (2.5-8.5) % vs. 1.8 (1.1-2.9) %, (P < 0.001 for the whole study population), while the expression of HLA-DR did not differ between CD8 $^+$ T cell subsets; 23 (13-36) % vs. 26 (19-34) %. As shown in Table 4, no differences in the expression of CD69 or HLA-DR were seen between patients and controls. The proportions of CD8 $^+$ CD56 $^+$ T cells coexpressing CD25 and CD94 were very low (< 0.5 %) in all groups.

The cytokine profile in CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells after PMA stimulation *ex vivo* was determined in 9 ACS patients, 14 SA patients and 11 controls. Within both CD8⁺ T cell subsets, the majority of cells were positive for IFN- γ and TNF without any difference between ACS patients, SA patients and controls. However, when the proportions of IFN- γ ⁺CD8⁺CD56⁺ T cells and IFN- γ ⁺CD8⁺CD56⁻ T cells were compared pair-wise, the proportions of IFN- γ ⁺CD8⁺CD56⁺ T cells were higher than the proportions of IFN- γ ⁺CD8⁺CD56⁻ T cells in all 24 individuals, 86 (71-92) *vs* 79 (75-85) %, p < 0.001. No significant difference was seen when comparing the proportions of TNF⁺CD8⁺CD56⁺ and TNF⁺CD8⁺CD56⁻ T cells, 65 (50-76) vs 61 (51-67) %. The proportions of IL-10, IL-13 and IL-17A expressing cells were low (< 1%) in both CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells, and showed no differences across groups.

Discussion

A major finding of the present study was the higher proportion of CD8⁺CD56⁺ T cells, constituting around 30 % of the CD8⁺ T cell population, in peripheral blood of CAD patients. In the age-matched control group, 21 % of the CD8⁺ T cells expressed CD56, which is in line with a study by Lemster et al¹⁰ documenting the similar proportions of CD8⁺CD56⁺ T cells in healthy 70-year old subjects. In patients, the expression of CD56 on CD8⁺ T cells seemed to be independent of clinical presentation since it was present in both ACS patients and SA patients. Moreover, the increase in CD8⁺CD56⁺ T cells persisted over time and was still detected in patients after one year independent of chosen therapeutic strategy (revascularization or conservative treatment). The increase in CD8⁺CD56⁺ T cells was not associated with hypertension, diabetes or number of diseased coronary vessels although it should be notified that the lack of associations (with e.g. diabetes) may be due to the small sample size. Systemic inflammatory activity, as assessed by IL-6, was increased at day 1 in both ACS and SA patients but declined during follow-up. At 12 months, IL-6 levels remained significantly elevated in SA patients but not in ACS patients, the latter possibly explained by the limited number of ACS patients during follow-up. Except for day 1 in ACS patients, CRP levels were generally low in patients probably reflecting the extensive use of statin. Several randomized placebo-controlled trials have consistently shown that a reduction of CRP is achieved by statin whereas the effect on cytokines, like IL-6, is more unclear ²⁵. The CD8⁺CD56⁺ T cell fractions did not correlate with CRP or IL-6 levels at any time point. Neither did the CD8⁺CD56⁺ T cell fraction correlate with plasma levels of IL-15, a cytokine specifically associated with CD8⁺CD56⁺ T cell survival in vitro²⁶. On the other hand, the correlation between CD8⁺CD56⁺ and CD4⁺ T cell numbers that was noticed in the patient population may illustrate a connection between CD56 expression and chronic immune stimulation. Previous cross-sectional studies have demonstrated the expansion of CD8⁺CD56⁺ T cells in other clinical settings of chronic immune stimulation, such as rheumatoid arthritis,

sarcoidosis and Behcet's uveitis^{11, 13-15}. In patients with active Behcet's uveitis, the higher proportions of CD8⁺CD56⁺ T cells were shown to decline after immunosuppressive treatment ¹⁶. Although it may be difficult to draw parallels between CAD and Behcet's uveitis due to differences in immunopathology and immune-based therapy, the findings by Ahn *et al* ¹⁶ indicate that the expansion of CD56 on CD8⁺ T cells is not necessarily a permanent phenomenon.

CMV is a common life-long infection, usually asymptomatic, that is believed to influence atherogenesis and cardiovascular risk²⁷. It is unique amongst herpesviruses by inducing senescence in T cells involving features like enhanced expression of natural killer cell markers and lack of CD28¹². Accordingly, we found increased proportions of CD8⁺CD56⁺ T cells in CMV seropositive individuals although CMV seropositivity alone could not explain the expansion of the CD8⁺CD56⁺ T cell subset in blood of CAD patients. Our finding is in agreement with a recent study by Romo *et al*²² reporting that proportions of CD56⁺ T cells were increased in patients with acute myocardial infarction compared with healthy controls, independent of age, sex, conventional risk factors and CMV seropositivity. Thus we now extend their finding by demonstrating that this phenomenon is not only restricted to patients with acute events, but also includes SA patients and furthermore that it persists for one year.

The early activation marker CD69 was more frequently expressed by CD8⁺CD56⁺ T cells than CD8⁺CD56⁻ T cells while the expression of HLA-DR did not differ. This is in agreement with a study by Kelly-Rogers et al²¹, showing that human CD8⁺CD56⁺ T cells were more prone to upregulate CD69 upon stimulation than were CD8⁺CD56⁻ T cells. The same authors showed that HLA-DR was equally induced on both CD8⁺ T cell subsets. As expected, we found a lack of CD28 on the majority of CD8⁺CD56⁺ T cells. The reciprocal expression of

CD56 and CD28 on peripheral T cells has been described by several^{10-11, 17} and is considered an indicator of T cell senescence. Resistance to apoptosis is considered another feature of senescent CD8⁺ T cells²⁸. We found significantly fewer apoptotic CD8⁺CD56⁺ T cells than apoptotic CD8⁺CD56⁻ T cells, the reduction being even more pronounced in the patient group. This may reflect a lower apoptotic rate of CD8⁺CD56⁺ T cells and thereby offer one plausible explanation for the accumulation of cells in the circulation. It has been shown in *vitro* that IL-15 not only induces the expression of CD56 on CD8⁺ T cells but also up-regulates the anti-apoptotic protein Bcl-2 making the cells more resistant to apoptosis²⁶.

The majority of both CD8⁺ T subsets showed capacity to produce IFN-γ and TNF. However, on an individual basis, the proportions of IFN-γ⁺ CD8⁺CD56⁺ T cells were always higher than the proportion of IFN-γ⁺ CD8⁺CD56⁻ T cells. This is in agreement with previous studies reporting that human blood-derived CD56⁺ T cells in culture produce larger amounts of IFN-γ than CD56⁻ T cells^{10, 15, 21}. Okhawa et al²⁰systematically compared CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells from healthy volunteers and showed significantly higher levels of IFN-γ in supernatants of stimulated CD8⁺CD56⁺ T cells. Since the proportions of CD8⁺CD56⁺ T cells were significantly increased in SA and ACS patients, it may be assumed that more IFN- γ^+ -producing cells are present in the circulation of patients. IFN- γ is considered one of the main effectors in the inflammatory response leading to atherosclerosis progression and plaque instability. Due to its short half-life, the cytokine is difficult to detect in plasma but an enhanced expression of IFN-y in circulating cells of CAD patients, in particular ACS patients, has been reported by several investigators⁵⁻⁶. Interestingly, plasma levels of cytokines belonging to the IFN-y axis have also been associated with increased mortality and morbidity after 1-year follow-up in both SA and ACS patients²⁹ and recently, a study by Pedersen et al³⁰showed that systemic markers of IFN-γ activity predicted long-term prognosis in SA

patients. It should be notified that in both these studies, as in our study, the majority of patients were treated with statin, a drug that is associated with T cell inhibitory effects³¹.

Negligible numbers of both CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells showed positive intracellular staining for IL-10, IL-13 and IL-17. The inability to produce IL-17 has been reported by others¹⁰ while the production of type 2 cytokines like IL-4 and IL-13, has been shown in some studies ^{10, 15, 21} but not others ²⁰. Similarly, there is an inconsistency with respect to IL-10 production. Kelly-Rogers et al²¹ did not find any secretion of IL-10 by CD56⁺ T cells derived from blood in healthy volunteers. On the other hand, CD56⁺ T cells in human colonic mucosal tissue have been shown to produce IL-10 and lower numbers of CD56⁺ T cells in the inflamed mucosa of ulcerative colitis compared with mucosa of healthy controls suggest an anti-inflammatory role of these cells 19 . This is in agreement with a study by Davila et al^{18} showing that CD8⁺CD28⁻CD56⁺ T cell clones generated from synovial tissue in patients with rheumatoid arthritis exerted anti-inflammatory effects in an experimental model of rheumatoid arthritis. When further characterized in vitro, CD56 was the most helpful marker in selecting CD8⁺CD28⁻ T cell lines with anti-inflammatory potential. Moreover, the identification of anti-inflammatory CD8⁺ T cells included the coexpression of CD25 and the natural killer receptor CD94. We therefore measured CD8⁺CD25⁺CD56⁺CD94⁺ T cells but found the numbers in blood to be negligible in both patients and controls. If we also take into account the inability to produce IL-10, there is no support for a major anti-inflammatory potential of CD8⁺CD56⁺ T cells in blood. However, it cannot be excluded that cells alter their functional phenotype when residing in other tissue compartments ¹⁸⁻¹⁹.

To summarize, an accumulation of CD8⁺CD56⁺ T cells in peripheral blood of ACS and SA patients was independently associated with the presence of CAD. The phenomenon appeared

to be persistent and may as such represent a state of immunological aging in CAD. The clinical relevance remains to be clarified, although it can be speculated that CD8⁺CD56⁺ T cells contribute to a larger IFN- γ ⁺ pool in blood thereby driving the atherosclerotic process in a less favorable direction.

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Table 1. Baseline characteristics of ACS patients, SA patients (at day 1) and controls.

	ACS patients	SA patients	Controls	<i>P</i> -value
	(n=30)	(n=34)	(n=36)	
Age, years	69 (60-78)	63 (58-71)	63 (58-73)	NS
Female, n (%)	8 (27)	6 (18)	9 (25)	NS
Current smokers, n (%)	7 (23)*	5 (15)	1 (2.8)	0.044
Waist circumference, cm	98 (93-109)*	102 (94-108)#	94 (88-99)	0.010
Hypertension, n (%)	11 (37)‡	24 (71)‡	0	< 0.001
Diabetes, n (%)	3 (10)	4 (12)	0	NS
0-1 vessel disease ^a , n (%)	13 (43)‡	9 (26)‡	0	< 0.001
2-3 vessel disease ^a , n (%)	17 (30)‡	25 (74)‡	0	< 0.001
Prior coronary event ^b , n (%)	4 (13)*	8 (24)#	0	0.010
Statin treatment, n (%)	9 (30)‡	28 (82)‡	0	< 0.001
Total cholesterol, mmol/l	5.2 (4.4-6.5)	5.0 (4.1-5.5)	5.6 (4.8-6.4)	NS
LDL cholesterol, mmol/l	3.2 (1.8-4.2)	2.7 (2.2-3.5)#	3.3 (2.8-4.0)	0.039
HDL cholesterol, mmol/l	1.3 (1.0-1.5)	1.2 (1.1-1.4)*	1.5 (1.2-1.8)	0.055
Triglycerides, mmol/l	1.4 (1.2-1.8)*	1.4 (1.1-1.9)*	1.1 (0.8-1.6)	0.043
CMV IgG seropositive, n (%)	22 (82)	29 (85)	26 (72)	NS

 $^{^{\}rm a}$ CAD determined by the number of significantly stenosed coronary arteries (significant stenosis defined as > 50 % diameter narrowing).

^b Prior coronary event: prior ACS and/or coronary revascularization.

^{*} P < 0.05 compared with controls, # P < 0.01 compared with controls

Table 2. The levels of CRP, IL-6 and IL-15 during the 12 months of follow-up.

		Day 1	3 months	12 months	<i>P</i> -value
CRP, mg/l	ACS	3.5 (2.2-5.0)‡	0.7 (0.3-1.9)	0.8 (0.3-2.8)	0.027
	SA	1.5 (0.4-3.5)	1.0 (0.3-3.2)	1.0 (0.3-2.2)	NS
	Controls	0.7 (0.4-1.2)	-	-	
IL-6, pg/ml	ACS	4.3 (2.2-7.7)‡	1.7 (1.2-3.1)	2.0 (1.4-3.1)	0.002
	SA	3.2 (2.0-6.5)‡	2.4 (1.2-3.0)*	2.5 (1.3-3.1)*	0.007
	Controls	1.3 (0.9-2.1)	-	-	
IL-15, pg/ml	ACS	3.1 (2.7-3.6)*	2.9 (2.4-3.3)	2.8 (2.6-3.3)	NS
	SA	2.9 (2.3-3.2)	2.6 (2.4-3.1)	2.9 (2.4-3.3)	NS
	Controls	2.6 (2.2-3.2)	-	-	

Differences within groups (from day 1 to 12 months) are given in the right column.

Table 3. Numbers and proportions of CD8⁺ and CD4⁺ T cell subsets

^{*} P < 0.05 compared with controls, ‡ P < 0.001 compared with controls

	ACS	SA	Controls	<i>P</i> -value
	(n = 30)	(n=34)	(n=36)	
Leukocytes, cells/µl	8067 (6173-9458)‡	6832 (5101-8398)#	5434 (4568-6417)	< 0.001
Lymphocytes, cells/µl	2319 (1706-2780)*	1996 (1308-2749)	1899 (1412-2307)	NS
CD8 ⁺ , cells/µl	593 (379-746)	449 (289-842)	416 (290-622)	NS
CD8 ⁺ CD56 ⁺ , cells/µl	173 (71-231)*	114 (65-275)*	78 (55-127)	0.021
% CD8 ⁺ CD56 ⁺ of CD8 ⁺	30 (18-35)*	29 (18-40)*	21 (13-28)	0.033
CD4 ⁺ , cells/μl	1210 (803-1557)‡	987 (612-1305)	819 (666-906)	0.002
CD4 ⁺ CD56 ⁺ , cells/μl	13 (4-64)	14 (7-29)*	6 (3-18)	0.091
% CD4 ⁺ CD56 ⁺ of CD4 ⁺	1.0 (0.4-4.8)	1.7 (0.8-4.0)	0.8 (0.4-2.3)	NS

^{*} P < 0.05 compared with controls, # P < 0.01 compared with controls, ‡ P < 0.001 compared with controls.

Table 4. Surface marker expression and apoptosis of CD8⁺ T cell subsets

	ACS	SA	Controls	<i>P</i> -value
	(n = 30)	(n=34)	(n=36)	
% CD28 of CD8 CD56 T cells	72 (46-84)	68 (44-78)	73 (46-90)	NS
% CD28 ⁻ of CD8 ⁺ CD56 ⁻ T cells	40 (26-63)	32 (16-62)	30 (13-51)	NS
% AnnexinV ⁺ of CD8 ⁺ CD56 ⁺ T cells	1.7 (0.2-3.2)*	1.8 (0.8-3.2)	2.9 (1.0-6.3)	0.040
% AnnexinV ⁺ of CD8 ⁺ CD56 ⁻ T cells	3.6 (2.4-6.2)	3.8 (2.8-7.3)	4.9 (3.0-7.0)	NS
% CD69 ⁺ of CD8 ⁺ CD56 ⁺ T cells	5.6 (2.5-11.3)	3.7 (1.8-7.5)	5.5 (3.4-8.8)	NS
% CD69 ⁺ of CD8 ⁺ CD56 ⁻ T cells	1.9 (1.1-2.5)	1.6 (0.9-3.6)	2.1 (1.2-3.2)	NS
% HLA-DR ⁺ of CD8 ⁺ CD56 ⁺ T cells	23 (13-40)	24 (13-34)	24 (14-37)	NS
% HLA-DR ⁺ of CD8 ⁺ CD56 ⁻ T cells	27 (20-34)	26 (17-34)	25 (20-37)	NS

^{*} P < 0.05 compared with controls