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Soluble Fas ligand is associated with natural killer cell dynamics in coronary artery disease

Aleksander Szymanowski[1], Wei Li[2], Anna Lundberg[1], Chamilly Evaldsson[1], Lennart Nilsson[1], Karin Backteman[3], Jan Ernerudh[3], Lena Jonasson[1]

[1]Department of Medical and Health Sciences, Division of Cardiovascular Medicine, Faculty of Health Sciences, Linköping University, SE-58185 Linköping, Sweden

[2]Department of Clinical and Experimental Medicine, Division of Experimental Pathology, Faculty of Health Sciences, Linköping University, SE-58185 Linköping, Sweden

[3]Department of Clinical and Experimental Medicine, Division of Clinical Immunology, Faculty of Health Sciences, Linköping University, Department of Clinical Immunology and Transfusion Medicine, County Council of Östergötland, SE-58185 Linköping, Sweden.

Corresponding author:

Lena Jonasson, Division of Cardiovascular Medicine, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköping University, SE-58185 Linköping, Sweden. Telephone: +46(0)10 103 51 94, Fax: +46(0)13 149 106, e-mail: lena.jonasson@liu.se

Abstract

Objective

Apoptosis of natural killer (NK) cells is increased in patients with coronary artery disease (CAD) and may explain why NK cell levels are altered in these patients. Soluble forms of Fas and Fas ligand (L) are considered as markers of apoptosis. Here, we investigated whether plasma levels of Fas and FasL were associated with NK cell apoptosis and NK cell levels in CAD patients.

Methods

Fas and FasL in plasma were determined by ELISA in 2 cohorts of CAD patients; one longitudinal study measuring circulating NK cells and apoptotic NK cells by flow cytometry 1 day, 3 months and 12 months after a coronary event and one cross-sectional study measuring NK cell apoptosis ex vivo. Both studies included matched healthy controls. Fas and FasL were also determined in supernatants from NK cells undergoing cytokine-induced apoptosis in cell culture.

Results

In the 12-month longitudinal study, plasma FasL increased by 15 % ($p<0.001$) and NK cell levels by 31 % ($p<0.05$) while plasma Fas did not change. Plasma FasL and NK cell levels were significantly related at 3 months and 12 months, $r = 0.40$, $p<0.01$. Furthermore, plasma FasL, but not plasma Fas, correlated with NK cell apoptosis ex vivo in CAD patients, $r =$

0.54, $p<0.05$. In vitro, cytokine-induced apoptosis of NK cells resulted in abundant release of FasL.

Conclusion

In CAD patients, FasL in plasma is associated with both apoptotic susceptibility of NK cells and dynamic changes in circulating NK cells. NK cells are also themselves a potential source of soluble FasL. Our findings link NK cell status to a soluble marker with possible atheroprotective effects thereby supporting a beneficial role of NK cells in CAD.

Keywords

Acute coronary syndrome; Coronary artery disease; Immune system; Leukocytes; Natural killer cells; Apoptosis

Introduction

Inflammation is considered an important component in the atherosclerotic process. It is present not only in the vessel wall but also in the circulation. Patients with coronary artery disease (CAD) show systemic signs of chronic low-grade inflammation but moreover, they exhibit a perturbed immune homeostasis involving circulating leukocytes (1,2). It has been shown by several, including our own group, that the circulating levels of natural killer (NK) cells as well as the NK cell activity are reduced in CAD patients compared with healthy subjects, the reduction being more pronounced in patients with unstable conditions (3-6). However, this is necessarily not a permanent feature. NK cells show a biological variation over time (7) and in a recently published 12-month follow-up study of patients with a coronary event, we demonstrated that NK cell levels could be restored to levels similar to controls (8).

The mechanism behind the deficit of NK cells in CAD is mainly unknown but an increased apoptosis of NK cells in CAD patients has been reported (5,9). Activation-induced lymphocyte apoptosis is largely mediated through the extrinsic pathway involving surface-bound receptors. One major pathway involves the death receptor Fas which induces apoptosis after interacting with the Fas ligand (FasL). Soluble forms of Fas (sFas) and FasL (sFasL) are detected in plasma and frequently used as markers of apoptosis. In neoplastic and autoimmune diseases, the levels of sFas and sFasL have been positively related to circulating levels of apoptotic NK cells and T cells (10-12). Interestingly, sFasL levels have also been positively associated with NK cell levels in NK cell proliferative disorders (13,14). In regard

to cardiovascular disease, high levels of sFas in plasma seem to indicate an increased risk (15-17) while elevations in sFasL rather represent a profile of low cardiovascular risk (15,18,19). The relationship between sFas or sFasL and lymphocyte apoptosis or lymphocyte levels in CAD is unknown.

Here, our aim was to investigate whether sFas and sFasL were positively related to NK cell apoptosis and NK cell levels in patients with acute and stable conditions of CAD, hypothesizing that these apoptotic markers could reflect the NK cell status in disease.

Methods

Subjects

Two independent cohorts of CAD patients (Cohorts I and II) were analysed. In Cohort I, 31 patients with non-ST-elevation acute coronary syndrome (NSTE-ACS) and 34 patients with stable angina (SA) were consecutively recruited from the Department of Cardiology, University Hospital, Linköping, Sweden. NSTE-ACS patients were included if they had a diagnosis of non-ST elevation myocardial infarction, with the diagnosis based on typical ECG changes (ST-T segment depression and/or T-wave inversion) and elevated troponins. SA patients were included if they were referred for elective coronary angiography due to effort angina class II or III in accordance with Canadian Cardiovascular Society Classification and objective signs of ischemia obtained by exercise testing or myocardial perfusion imaging. Blood samples at day 1 were collected immediately before coronary angiography and in

NSTE-ACS patients within 24 h from admission. Longitudinal measures were available in 15 NSTE-ACS patients and 28 SA patients, blood samples being collected at 3 and 12 months.

In Cohort II, 16 CAD patients who had suffered a coronary event (NSTE-ACS and/or coronary revascularization procedure) 3-6 months earlier were consecutively recruited from the same cardiology department as Cohort I. Patients in Cohort II were recruited before the inclusion in Cohort I started and none of the patients were included in both cohorts.

Patients were not eligible in any of the cohorts 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).

In parallel with the recruitment of Cohort I and II, age and gender matched control subjects from the region were randomly invited from the Swedish Population Register. None of the controls participated in both studies. Subjects who accepted the invitation were included as controls if they were anamnestically healthy and received no medication. Anamnesis was gained by one dedicated nurse coordinator who also performed the blood sampling at the University Hospital´s Outpatient Cardiology Clinic.

The studies were conducted in accordance with the ethical guidelines of Declaration of Helsinki, and the research protocols were approved by the Ethical Review Board of Linköping University. Written informed consent was obtained from all patients and controls before blood collection.

Whole blood flow cytometric analysis of NK cells and T cells and their levels of apoptosis

In Cohort I, circulating levels of NK cells and T cells as well as apoptotic NK cells and T cells were assessed by whole blood flow cytometry within 1 hour from blood sampling. Cells were identified by using the following monoclonal antibodies: anti-CD3-fluorescein isothiocyanate (FITC) and anti-CD56-allophycocyanin (APC), both purchased from BD Biosciences (San José, CA, USA). As previously described (6, 8), whole blood and antibodies were incubated for 15 minutes at room temperature, erythrocytes were thereafter lysed with FACS™ Lysing Solution (BD Biosciences) for 15 minutes at room temperature. For the detection of apoptosis/necrosis, cells were resuspended in 100 μl of annexin V binding buffer and incubated with 3.5 μl of phycoerythrin (PE)-conjugated annexin V and 3.5 μl of peridinin chlorophyll protein (PerCP)-conjugated 7-aminoactinomyosin D (7-AAD) at room temperature for 15 minutes in the dark (all reagents from BD Biosciences). Next, an aliquot of 400 μl annexin V binding buffer was added and flow cytometry analysis was performed immediately. Samples were analyzed on a FACSCanto A or FACSCanto II (BD Biosciences). 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). Control of the instrument settings was done daily with 7-color Setup Beads™ with FACSDiva™ software or Cytometer Setup and Tracking beads™ (BD Biosciences) with Cytometer Setup and Tracking™ software according to the standard procedure. The flow cytometry laboratory gained accreditation status in 1996 according to ISO/IEC 17025 standards and is participating in external quality programs for immune phenotyping (UK Neqas and Equalis). The methodological and biological variation in lymphocyte onehotyping has been reported previously (7).

Preparation of NK cells and T cells

In order to perform apoptosis assays ex vivo and in vitro, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. After centrifugation at 400 x g for 30 min, the PBMC were collected at the interface and washed twice. For the assessment of lymphocyte apoptosis ex vivo, NK cells and T cells were derived from patients and controls in Cohort II by using NK Cell Negative Isolation Kit (Dynal Biotech ASA, Oslo, Norway) as previously described (9). For the assessment of cytokine-induced apoptosis in vitro, NK cells and T cells were derived from blood donors by using stepwise isolation of CD3+ and CD3- cells and subsequent positive selection of CD3-CD56+ cells using MACS beads following the manufacturer's protocol (Milteney Biotec, Bergich Gladbach, Germany). All selected cell fractions were analysed by flow cytometry using anti-CD3-APC-H7 and anti-CD56-APC (BD Biosciences). Only preparations with > 97 % CD3-CD56+ cells (NK cells) or > 97 % CD3+ cells (T cells) were used for further experiments.

Spontaneous NK cells and T cell apoptosis ex vivo

In Cohort II, the susceptibility to apoptosis of NK cells and T cells was determined in ex vivo assays. Blinded analyses were performed involving one patient and one age- and gender-matched control in each experimental set-up. Fractions of isolated NK cells or T cells were cultured in RPMI culture medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 µg/mL penicillin G and 100 µg/mL streptomycin. The cells were cultured at 95 % air and 5 % CO_2 in a humidified atmosphere at 37º C for 18 hours. Thereafter, cells were collected, washed once and stained for 10 minutes on ice with annexin V-FITC and propidium iodide (Roche Diagnostics GmbH, Mannheim, Germany). Finally, samples were analysed on Becton-Dickinson LSR II Flow Cytometer using CellQuest software (10 000 cells were analysed in each sample). The number of apoptotic cells were also measured directly after

isolation and used as a baseline reference. Quality assurance procedures using standard operating practice and Spherotech 8-peak validation beads (Becton Dickinson) every second week ensured reproducibility.

Cytokine-induced apoptosis of NK cells and T cells in vitro

The ability of NK cells and T cells to secrete Fas or FasL upon cytokine-induced apoptosis was determined in vitro. NK cell and T cell fractions were isolated from 5 healthy blood donors (according to the procedure described above), plated at 100 000 cells per well in 96-well plates and cultured at 37º. NK cells or T cells were either left unstimulated, stimulated with interleukin (IL)-15 alone (15 ng/ml, R&D Systems, UK) or with IL-15 and IL-12 p70 (3 ng/ml, R&D Systems, UK) according to a protocol by Ross et al (20). After 48 h of stimulation, supernatants were collected. Apoptosis was determined by flow cytometry after staining with APC-conjugated annexin V and antibodies against CD3 (APC/H7) and CD56 (FITC).

Measurements of Fas, FasL and interleukin-6 in plasma and after stimulation in vitro

Concentrations of Fas and FasL were determined in plasma samples from Cohort I and II and in cell supernatants after in vitro release by using Quantikine® ELISA kit (R&D systems, Abingdon, UK) following the manufacturer´s instructions. Quantifications of IL-6 were done in plasma samples from Cohort I by using QuantiGlo® Chemiluminescent ELISA (R&D systems) following the manufacturer´s instructions and the plates were read in a luminometer (Biotek, Germany). The limits of detection were as follows; Fas and FasL both 7.8 pg/mL and IL-6 0.48 pg/mL. Interassay coefficients or variation were < 3 %, < 10 % and < 5 % for Fas,

FasL and IL-6, respectively. Samples were analyzed in duplicate with an intra- assay coefficient of variance for Fas, FasL and IL-6 of 7.5 %, < 8.0 % and < 15 %, respectively.

Statistical analyses

IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The Pearson´s chi-square test and Mann-Whitney U-test were used for analyses between two groups and Kruskal-Wallis one-way analysis of variance for comparison between 3 groups. The differences between related samples were analysed by Friedman´s test. Bivariate correlations were analysed by Spearman´s rank correlation coefficient. Statistical significances were set at a two-tailed p-value of < 0.05. Values are given as median (inter-quartile range).

Results

Plasma levels of sFasL are associated with recovery of NK cell levels in CAD patients (Cohort I)

Clinical and laboratory characteristics of Cohort I with age and gender matched controls are summarised in Table 1. Compared with controls, the prevalence of smoking was higher in NSTE-ACS and SA patients. Also, the waist circumference was larger in both patient groups. None of the controls were treated with antihypertensive drugs or statin. In the SA group, LDL cholesterol levels were lower than in NSTE-ACS and controls while triglyceride levels were higher in both patient groups. Also, IL-6 levels were higher in NSTE-ACS and SA patients than in controls, 4.3 (2.2-7.7), 3.2 (2.0-6.5) and 1.4 (1.0-2.2) pg/ml, respectively, p < 0.001.

The levels of sFas, sFasL and subsets of NK and T cells are presented in Table 2. At day 1, the levels of sFas were similar across groups while sFasL levels were lower in NSTE-ACS patients compared with SA patients and controls. In the total lymphocyte population, the proportions of NK cells were lower in both NSTE-ACS and SA patients compared with controls. In the NK cell population, the proportions of early apoptotic (annexin V+/7-AAD-) cells were low in all groups (Table 2). Late-stage apoptotic or already dead lymphocyte subsets (annexin V+/7-AAD+) were not detectable in any blood samples.

In total, 43 patients in Cohort I (15 NSTE-ACS and 28 SA) participated in the longitudinal study. Among them, 33 (77 %) had undergone coronary revascularization while 10 (23 %) had obtained optimized anti-anginal drug therapy. During follow-up, all patients received treatment with statin. The longitudinal changes of sFas, sFasL, IL-6 and subsets of NK and T cells are shown in Table 3. Over 12 months, the levels of sFasL showed a relative increase by 17 (2.0-39) % in the NSTE-ACS group and 15 (0.0-30) % in the SA group, p for both < 0.01. In all 43 patients, the relative increase in sFasL during 12-month follow-up was 15 (0.0-32) %, $p < 0.001$. Over the same period, the relative increase in NK cells was 35 ((-1.5)-57) % in the NSTE-ACS group and 24 ((-6.8)-75) % in the SA group, $p < 0.01$ and < 0.05, respectively. In all 43 patients, the increase in NK cells during 12-month follow-up was 31 ((-4.0)-57) %, $p < 0.05$. At day 1, there were no correlations between sFas or sFasL on one hand, and NK cells or apoptotic NK cells on the other hand, neither in patients nor in control subjects. Instead, positive correlations were seen between sFasL and proportions of NK cells in patients during follow-up, both at 3 months ($r = 0.40$, $p < 0.01$, see Figure 1) and 12 months ($r = 0.43$, $p < 0.01$). Weaker correlations were also seen between sFasL and absolute numbers of NK cells at 3 months ($r = 0.28$, $p < 0.05$) and 12 months ($r = 0.31$, $p = 0.06$) and between relative increase in NK cells and relative increase in sFasL at 3 months ($r = 0.31$, $p <$

0.05). In bivariate correlation analyses, using significance level of < 0.01, the proportions of NK cells or plasma levels of FasL were not correlated with age, gender, smoking, or waist circumference. Neither were NK cell levels or plasma sFasL correlated with plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, cystatin C, IL-6 or sFas at any time point.

Plasma levels of sFasL are associated with increased susceptibility to NK cell apoptosis in CAD patients (Cohort II)

All patients in Cohort II were recruited 3-6 months after a coronary event thus being in a similar clinical stage as Cohort I during follow-up. In pairwise experiments, spontaneous apoptosis of freshly isolated NK cells and T cells was compared in 16 patients and 16 age and gender matched healthy controls. Besides a brief summary of participants´ clinical characteristics, Table 4 shows the plasma levels of sFas and sFasL and the apoptotic susceptibility of NK cells and T cells. The levels of sFas or sFasL did not differ between groups. NK cells from patients were significantly more susceptible to apoptosis compared with NK cells from controls. T cells, on the other hand, were less prone to apoptosis compared with NK cells with no difference between groups. In patients, the levels of sFasL were significantly correlated with susceptibility to NK cell apoptosis ($r = 0.54$, $p < 0.05$, see Figure 2), whereas no such correlation was seen in controls ($r = 0.08$, NS). There was no correlation between sFasL and apoptosis of T cells, neither between sFas and apoptosis of NK cells or T cells.

NK cells undergoing apoptosis are a major source of FasL

To further evaluate the association between sFasL and apoptosis, NK cells and T cells from blood donors were cultured under stimulatory conditions to induce apoptosis. After 48 h stimulation with IL-15, NK cells showed increased size, granularity and CD56 expression (as indicators of activation) but also increased apoptosis compared with unstimulated cells (Figure 3). Stimulation with a combination of IL-15 and IL-12 resulted in a larger proportion of apoptotic NK cells compared with IL-15 alone. T cells, on the other hand, were less prone to enter apoptosis both spontaneously and in the presence of IL-15 and/or IL-12 (data not shown). As shown in Figure 4, the concentrations of sFasL increased markedly in supernatants from NK cells stimulated with IL-15 compared with medium alone and increased even more, up to 20-fold, when NK cells were stimulated with IL-15 and IL-12. Compared with NK cells, T cells showed a less pronounced increase in sFasL upon cytokine stimulation. The concentrations of sFas in non-stimulated supernatants were low but NK cell release of sFas increased around 5-fold and T cell release of sFas around 2-fold upon stimulation (data not shown).

Discussion

In the present study, we provide evidence for a dynamic relationship between sFasL and NK cells in CAD patients. The novel findings can be summarized as follows: 1) plasma levels of sFasL were associated with recovery of NK cell levels in patients after a coronary event, 2) sFasL in plasma was associated with increased susceptibility to NK cell apoptosis in CAD patients and 3) apoptotic NK cells were themselves found to be a source of FasL.

The reduction of NK cells in CAD patients is well documented (3-6) and has been attributed to increased apoptosis of these cells (5, 9). However, the increase in circulating NK cells that occurs in CAD patients after a coronary event (8) may also indicate an increased proliferation of these cells. A balance between apoptotic and proliferative rates is critical for maintaining homeostasis and normally, increased proliferation of cells is linked to increased apoptosis. Although FasL is best known for its death-inducing function and as such, is considered a marker of apoptosis, it also has a role as signaling receptor in proliferation (21-23). The association between sFasL and NK cell recovery in patients after a coronary event suggests that sFasL not only reflects an increased apoptotic rate but rather an increased apoptotic/proliferative rate of NK cells. Interestingly, previous studies have shown that plasma levels of sFasL are increased in NK cell proliferative disorders but not in other lymphoproliferative disorders like T cell leukemia or T and B cell lymphoma (13,14). Furthermore, the high sFasL levels in NK cell proliferative disorders become normalized after chemotherapy when NK cell levels decline (14).

In the present study, there were no correlations between sFas or sFasL and circulating apoptotic lymphocytes in CAD patients at any time point. Such correlations have been reported in gastric cancer (10,11) as well as in systemic lupus erythematosus (12). In the studies of gastric cancer, remarkably high proportions of apoptotic NK cells and CD8+ T cells in blood were reported, 20 % in patients and 10 % in healthy controls (10,11). In the study by Courtney et al (12), 4 % of lymphocytes were apoptotic in patients with systemic lupus erythematosus compared with 1 % in controls. Since apoptosis is a time-sensitive process, we always performed flow cytometry within 1 hour from blood sampling. We found low numbers of apoptotic NK cells and T cells in both CAD patients and controls, similar to levels that were reported in healthy controls by Courtney et al (12). However, when apoptosis was

assessed ex vivo, we found that NK cell apoptosis was increased in CAD patients compared with controls. Moreover, susceptibility to NK cell apoptosis was significantly associated with plasma sFasL in patients, but not in control subjects, supporting the hypothesis that sFasL is associated with NK cell apoptosis under disease conditions.

Both NK cells and cytotoxic T cells are potential sources of sFasL (21, 22, 24). To further evaluate this, we isolated human NK cells and T cells and compared their capacity to release FasL upon cytokine-induced apoptosis. In the 48-hour stimulation assay, the NK cell release of FasL was particularly abundant indicating that apoptotic NK cells themselves may be a prominent source of sFasL.

In previous studies, sFasL has emerged as a possible atheroprotective marker. Blanco-Colio et al (19) showed a positive correlation between sFasL levels and forearm reactive hyperemia in patients with CAD speculating that normal endothelial cells contributed to circulatory levels of sFasL. Furthermore, low levels of sFasL have been reported in patients with manifest CAD as well as in individuals at high cardiovascular risk (17, 18). FasL is cleaved from the cell membrane to produce a soluble form. In contrast to membrane-bound FasL, the soluble form has been associated with anti-apoptotic and anti-inflammatory effects. In experimental settings, the soluble form is 1000-fold less active than the membrane-bound form (22) and moreover, it has been shown to antagonize the effects of membrane-bound FasL by competitive binding to Fas without inducing apoptosis (25). It may be tempting to speculate that an increase in sFasL is needed to counteract enhanced apoptosis of NK cells in order to raise and/or maintain the levels of NK cells. Our results indicate that NK cells themselves are a potential source of sFasL in the circulation. During the cardiac rehabilitation phase, it is also possible that sFasL is released by endothelial cells as an indicator of improved endothelial

function (19). Improved endothelial function is associated with an improved inflammatory state, which in its turn may raise NK cell levels. Based on experimental studies, an inflammatory milieu has been associated with inhibited NK cell development and shortened survival of NK cells (26).

The descriptive study design is one limitation of our study. Another limitation is the small size of study populations. We were thus not able to investigate the impact of possible confounders, such as smoking, hypertension or diabetes, on sFas or sFasL levels. On the other hand, the patient cohorts recruited at one single site were well characterized and treated according to current guidelines. The combined use of longitudinal analyses of circulating cells and ex vivo analyses may also be considered a strength.

To summarize, we show for the first time that sFasL in plasma is associated with an increased susceptibility to NK cell apoptosis in CAD patients and also, that an increase in sFasL following treatment of a coronary event is associated with an increase in circulating NK cells. Our findings link NK cell status to a soluble marker with possible atheroprotective effects and may thereby support a beneficial role for NK cells in CAD. It remains to be confirmed in larger studies of CAD patients that sFasL is useful to monitor changes in NK cell levels. Also, it needs to be evaluated in prospective studies whether an increase of sFasL after a coronary event is a good prognostic sign.

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Figure legends

Figure 1. The correlation between sFasL levels and proportions of NK cells (% of all lymphocytes) in CAD patients 3 months after a coronary event (Cohort I), $r = 0.40$, $p < 0.01$.

Figure 2. The correlation between sFas levels and NK cell apoptosis ex vivo (shown as fold increase compared to baseline) in CAD patients 3-6 months after a coronary event (Cohort II), $r = 0.54$, $p < 0.05$.

Figure 3. One illustrative example of morphological and phenotypical changes of purified natural killer (NK) cells derived from blood donors. Cells were cultured for 48 hours in medium only or in the presence of interleukin (IL)-15 or IL-12 + IL-15 Activated cells were defined by increase in cell size accompanied by a modest increase in granularity compared with resting cells. Moreover, activated cells showed a higher expression of CD56 ($CD56^{bright}$ cells). Apoptotic cells, on the other hand, were defined by decrease in cell size and increased granularity compared with resting cells. Apoptosis was also confirmed by Annexin V staining in parallel experiments. Compared with medium alone, incubation with IL-15 increased the proportions of activated and apoptotic NK cells. It also increased the proportion of $CD56^{bright}$ cells. Incubation with IL-12+IL-15 resulted in more apoptotic NK cells compared with IL-15 alone. The lower proportions of activated NK cells as well as $CD56^{bright}$ cells after incubation with IL-12+IL-15 was probably due to increased activation-induced apoptosis.

Figure 4. Release of Fas ligand (L) by purified subsets of natural killer (NK) cells (CD3-56+) and T cells (CD3+) derived from blood donors. Fractions of 100 000 cells were cultured in

medium alone, IL-15 (15 ng/mL) or IL-15 + IL-12 (15 and 3 ng/mL respectively) for 48 h in 37°C. Thereafter supernatants were collected and analysed for FasL, n=5.

Table 1. Baseline characteristics of NSTE-ACS patients and SA patients in Cohort I (day 1 prior to coronary angiography) and clinically healthy controls.

	NSTE-ACS (n = 31)	SA (n = 34)	Controls (n = 37)	p[a]
Age, years	69 (50-83)	63 (44-77)	63 (45-77)	0.155
Female, n (%)	8 (26)	6 (18)	9 (24)	0.697
Smokers, n (%)	7 (23) [d]	5 (15) [d]	1 (3)	**<0.001**
Waist circumference (cm)	98 (93-108) [b]	102 (94-108) [c]	94 (89-99)	**0.011**
Hypertension, n (%)	12 (39) [d]	24 (71) [d]	0	**<0.001**
Diabetes, n (%)	3 (10)	4 (12)	0	0.111
Statin treatment, n (%)	9 (29) [d]	28 (82) [d]	0	**<0.001**
Total cholesterol, mmol/l	5.3 (4.4-6.4)	5.0 (4.1-5.5)	5.5 (4.9-6.4)	0.157
LDL cholesterol, mmol/l	3.3 (1.9-4.1)	2.7 (2.2-3.5) [c]	3.4 (2.8-4.0)	**0.030**
HDL cholesterol, mmol/l	1.3 (1.0-1.5)	1.2 (1.1-1.4) [b]	1.4 (1.2-1.8)	0.064
Triglycerides, mmol/l	1.4 (1.2-1.8) [b]	1.4 (1.1-1.9) [b]	1.1 (0.9-1.6)	**0.026**
Cystatin C, mg/l	1.1 (1.0-1.3)	1.1 (1.0-1.2)	1.1 (1.1-1.2)	0.659
IL-6, pg/ml	4.3 (2.2-7.7) [d]	3.2 (2.0-6.5) [d]	1.4 (1.0-2.2)	**<0.001**

NSTE-ACS, non-ST-elevation acute coronary syndrome; SA, stable angina. Ordinal data are presented as median (inter-quartile range). [a] Kruskal-Wallis one-way analysis of variance was used for comparison between 3 groups. Mann Whitney U-test was used for comparison between two groups, [b] $p < 0.05$ compared with controls, [c] $p < 0.01$ compared with controls, [d] $p < 0.001$ compared with controls.

Table 2. Soluble markers of apoptosis, proportions of NK cells and T cells (% of lymphocytes) and proportions of early apoptotic NK and T cell subsets (% of NK or T cells) in NSTE-ACS and SA patients in Cohort I (day 1 prior to coronary angiography) and clinically healthy controls.

	NSTE-ACS (n = 31)	**SA** (n = 34)	**Controls** (n = 37)	p[a]
sFas, pg/ml	7308 (6235-8364)	7010 (6347-8134)	7546 (6414-8611)	0.785
sFasL, pg/ml	49 (35-52) [b, d]	54 (39-64)	52 (43-74)	**0.049**
NK cells, %	12 (9.8-15) [b]	12 (9.1-18) [b]	18 (11-28)	**0.020**
T cells, %	76 (68-80) [c]	75 (70-80) [c]	65 (60-74)	**0.001**
Apoptotic NK cells, %	0.4 (0.2-0.8)	1.0 (0.4-1.8)	0.6 (0.4-2.1)	0.906
Apoptotic T cells, %	0.5 (0.3-1.1)	1.3 (0.6-1.8)	0.7 (0.4-2.2)	0.372

Soluble, s; NSTE-ACS, non-ST-elevation acute coronary syndrome; SA, stable angina; Fas L, Fas ligand; NK cell, natural killer cell. Ordinal data are presented as median (inter-quartile range). [a]Kruskal-Wallis one-way analysis of variance was used for comparison between 3 groups. Mann Whitney U-test was used for comparison between two groups, [b] $p < 0.05$ compared with controls, [c] $p < 0.01$ compared with controls, [d] $p < 0.05$ compared with SA patients.

Table 3. Plasma levels of sFas, sFasL and IL-6, proportions of NK and T cells (% of lymphocytes) and proportions of early apoptotic NK cells and T cells (% of NK or T cells) in a longitudinal analysis of CAD patients with a coronary event; day 1 (prior to coronary angiography), 3 months and 12 months (Cohort I, n = 43, 15 NSTE-ACS, 28 SA).

	Day 1	**3 months**	**12 months**	**p**[a]
sFas, pg/ml	7275 (6332-8279)	7849 (6451-8790)	7746 (6848-8869)	0.689
sFasL, pg/ml	51 (37-60)	55 (42-63)	57 (43-70)	**<0.001**
IL-6, pg/ml	3.8 (2.1-6.5)	2.2 (1.2-3.1)	2.2 (1.3-3.1)	**0.002**
NK cells, %	12 (8.6-16)	14 (9.7-20)	16 (11-20)	**0.011**
NK cells/µl	250 (142-371)	291 (200-375)	300 (237-448)	**<0.001**
T cells, %	76 (69-80)	75 (69-80)	74 (68-78)	0.064
T cells/µl	1700 (990-2128)	1415 (995-2144)	1590 (1070-2115)	0.159
Apoptotic NK cells, %	0.5 (0.2-1.3)	1.1 (0.5-2.2)	0.9 (0.5-1.2)	0.687
Apoptotic T cells, %	1.0 (0.5-1.6)	1.4 (0.6-2.5)	0.7 (0.4-1.4)	0.146

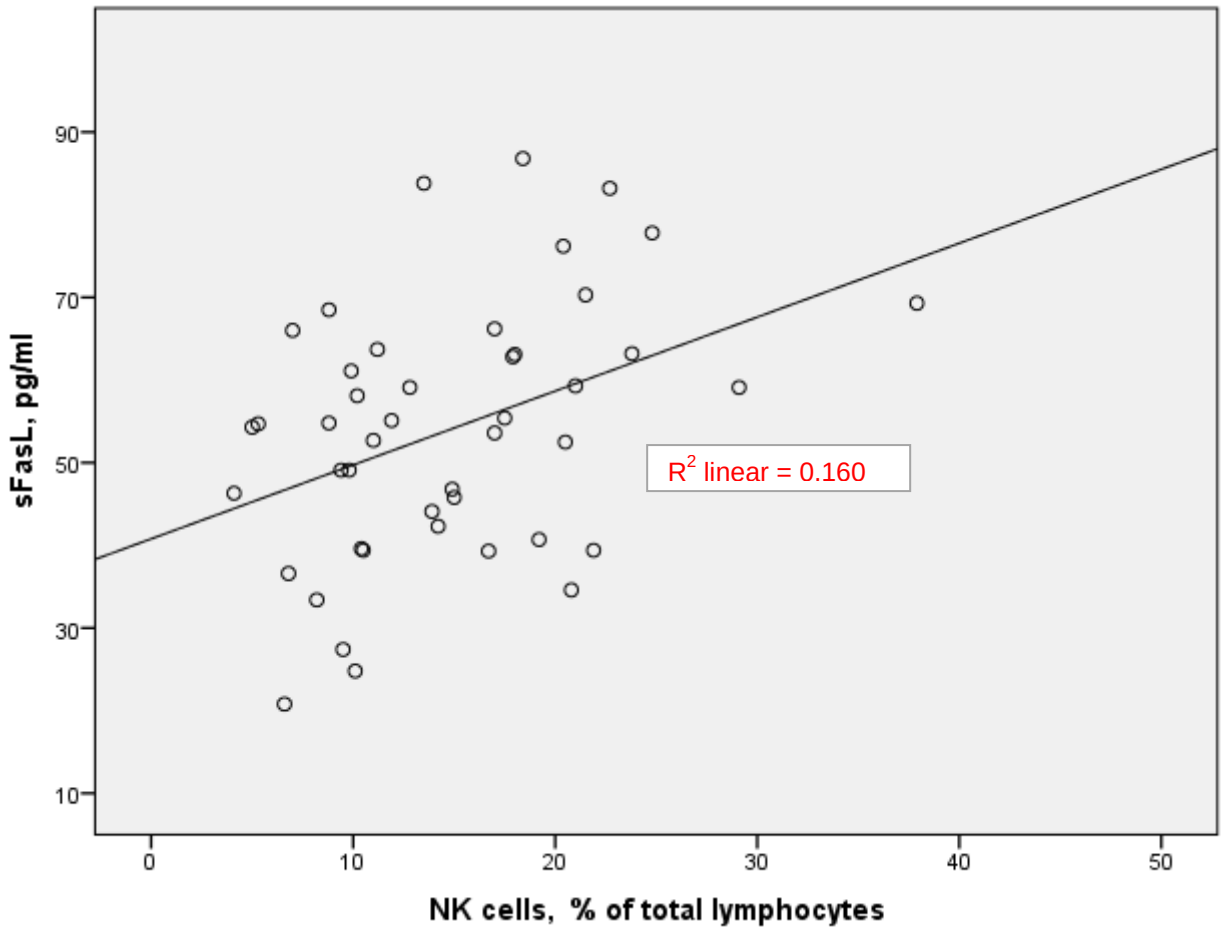
Soluble, s; Fas L, Fas ligand; interleukin-6, IL-6; NK cell, natural killer cell. Ordinal data are presented as median (inter-quartile range). [a]The differences over time were analysed by Friedman´s test.

Table 4. Clinical characteristics, plasma levels of sFas and sFasL and ex vivo apoptosis of NK cells and T cells in 16 matched pairs of CAD patients 3-6 months after a coronary event (Cohort II) and clinically healthy controls.

	Patients n = 16	Controls n = 16	p[a]
Age, years	60 (58-65)	60 (55-62)	0.210
Female, n (%)	2 (14)	2 (14)	1.000
Smokers, n (%)	2 (14)	2 (14)	1.000
Body mass index, kg/m^2	25 (24-30)	25 (24-38)	0.569
sFas, pg/ml	8087 (6932-8845)	7187 (6261-8035)	0.216
sFasL, pg/ml	55 (48-66)	57 (48-63)	0.926
NK cell apoptosis	2.52 (1.75–3.37)	2.00 (1.35-2.77)	**0.024**
T cell apoptosis	0.87 (0.79-1.05)	0.65 (0.55-0.97)	0.220

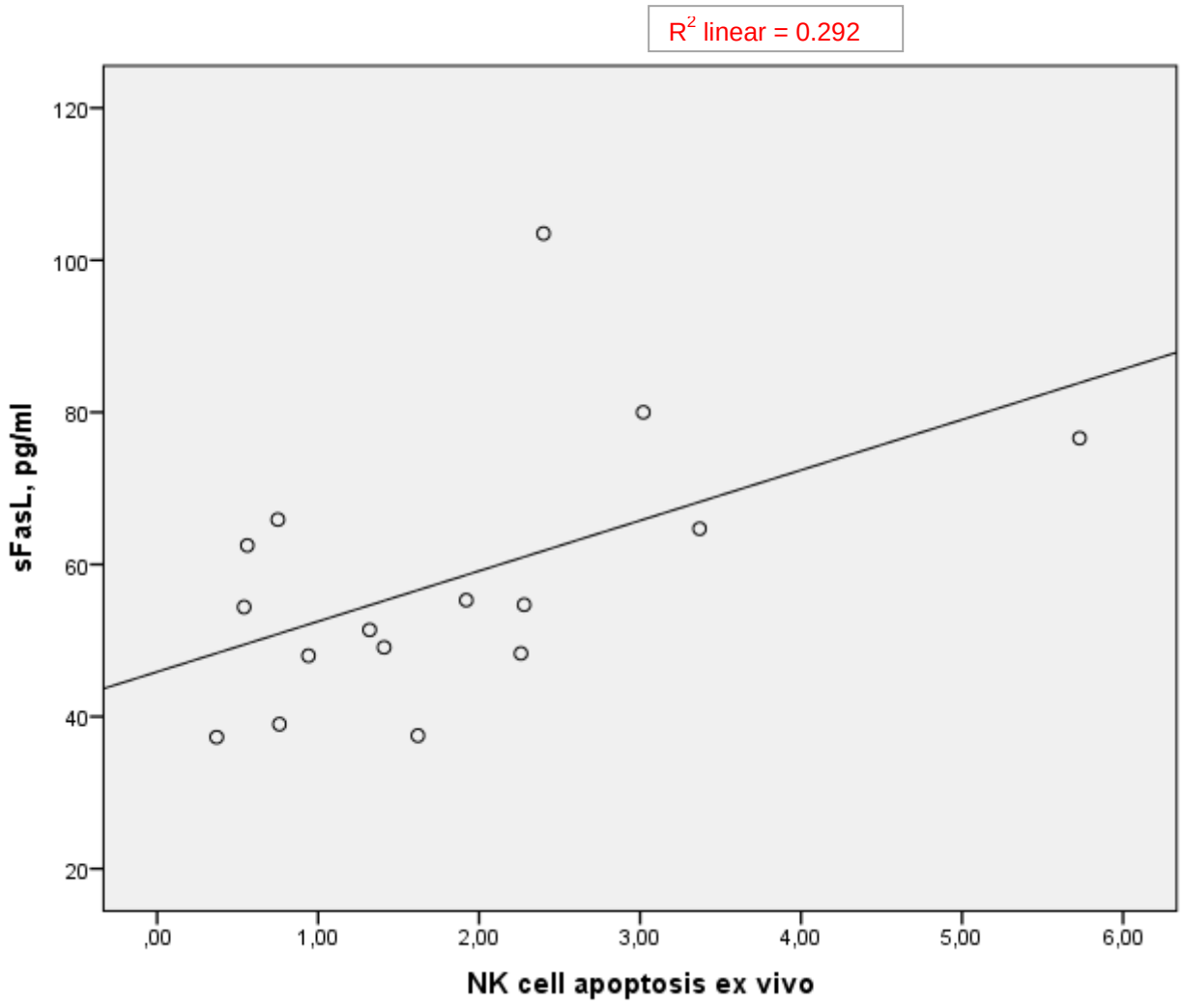
NSTE-ACS, non-ST-elevation acute coronary syndrome; SA, stable angina; soluble, s; Fas L, Fas ligand; NK cell, natural killer cell. Ordinal data are presented as median (inter-quartile range). Ex vivo apoptosis of NK cells and T cells is presented as fold increase compared to baseline. [a]Mann Whitney U-test was used for comparison between the groups.

Figure 1.



Fas L, Fas ligand; NK cell, natural killer cell

Figure 2.


R² linear = 0.292
sFasL, pg/ml
120
100
80
60
40
20
,00
1,00
2,00
3,00
4,00
5,00
6,00
NK cell apoptosis ex vivo

Fas L, Fas ligand; NK cell, natural killer cell

Figure 3.