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CD4+CD56+ NKT-like cells secreting interferon-γ are associated with incident coronary events

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Running title: NKT-like cells and incident MI

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Abstract

**Background:** CD3+CD56+ NKT-like cells are a subset of T cells characterized by expression of NK receptors and potent antitumor activity. They have also been implicated in autoimmune disease and shown to be elevated in patients with coronary disease.

**Objectives:** To investigate if high levels of CD3+CD56+ NKT-like cells are associated with an increased incidence of cardiovascular disease and a lower incidence of cancer.

**Methods:** A prospective study including 700 subjects participating in the baseline investigation of the Malmö Diet and Cancer Study between 1991 and 1994. Leukocytes obtained at the baseline investigation and stored at -140°C were thawed and CD3+CD56+ cells analyzed by flow cytometry. The incidence of cancer and coronary events during a mean follow-up of 15 years were obtained through national registers.

**Results:** Subjects in the lowest tertile of IFN-γ expressing CD4+CD56+ cells were found to have an increased risk for incidence of coronary events (log-rank test: $P <0.05$). This association remained significant when controlling for age, sex, smoking, BMI, hypertension, diabetes, the Th1/Th2 and the Th1/Treg cell ratio in a Cox proportional hazards regression model (hazard ratio 1.98, 95% C.I. 1.24-3.16), but not when LDL/HDL ratio was included in the model. There were no associations between CD3+CD56+ NKT-like cells and incident cancer.

**Conclusions:** The present study could not confirm the hypothesis that low levels of CD3+CD56+ NKT-like cells are associated with a higher incidence cancer and a lower incidence of CVD. Contrarily, we found that low levels of IFN-γ expressing CD3+CD4+CD56+ NKT-like cells was associated with an increased incidence of coronary events and that this association may be dependent on lipoproteins.
Key words: NKT-like cells, cardiovascular disease, cancer
Introduction

CD3+CD56+ T cells belong to a family of T cells that express NK markers but in contrast to the classical invariant NKT cells they are not CD1d-alphaGalCer restricted and are therefore referred to as NKT-like cells [1]. NKT-like cells have a more diverse T cell repertoire than invariant NKT cells and are found mainly in the spleen and in the bone-marrow [2]. They are rare early in life but increase with age and constitute about 20% of all CD8+ T cells and about 5% of all CD4+ T cells by the age of 80 years [3]. CD3+CD56+ T cells expand in response to virus infection [4] and release cytokines that stimulate the recruitment of innate immune cells [5]. CD3+CD56+ T cells have been shown to possess antitumor activity [6] and have been postulated to play a role in tumor surveillance [7]. High levels of CD3+CD56+ T cells are associated with improved survival in chronic lymphatic leukemia, lung and colon cancer [8, 9]. Moreover, clinical trials using ex vivo expanded CD3+CD56+ T cells (so called cytokine-induced killer cells) have demonstrated encouraging results both in hematological malignancies and solid tumors [10]. However, increased levels of CD3+CD56+ T cells have been also reported in several autoimmune diseases including rheumatoid arthritis, sarcoidosis and Behcet’s uveitis [11-13] and the expression of CD56 on T cells has been shown to correlate with a loss of CD28, a hallmark of emerging immune-senescence [14] Bergström et al recently [15] reported increased levels of CD3+CD56+ T cells in patients with coronary artery disease and demonstrated that these cells were characterized by down-regulation of CD28 and an increased expression of interferon (IFN)-γ. Since the atherosclerotic disease processes has been shown to be modulated by adaptive immune responses [16] it is an interesting possibility that this finding could reflect a diminished ability to control autoimmune responses against oxidized LDL and other self-antigens in atherosclerotic plaques. Against this background it could be hypothesized that high levels of CD3+CD56+ NKT-like cells would
be associated with a lower risk of cancer as well as with a higher incidence of coronary events (CE). To test this hypothesis we used mononuclear cells from the Malmö Diet and Cancer (MDC) study biobank to study the association of CD3+CD56+ T cells with incident cancer and CE during 15 years of follow-up.

**Material and methods**

*Study population*

The study cohort, consisting of 700 participants aged 63–68 years from the MDC cardiovascular cohort has been previously described [17]. Briefly, participants were followed from baseline examination in 1991-1994 until first CVD or cancer event, emigration from Sweden, death or 31 December 2008, whichever came first. A CVD event was defined as a fatal or nonfatal myocardial infarction [i.e. International Classification of Diseases, 9th Revision (ICD-9) code 410], fatal or non-fatal stroke (ICD-9 codes 430, 431, 434 and 436) or death attributable to underlying coronary heart disease (ICD-9 codes 410–414). A total of 150 incident CVD events (84 coronary events and 66 strokes) were identified during the follow-up period. Classification of cancer was based on ICD-9 codes 140-239. A total of 170 incident cancer events were identified during the follow-up period. Hypertension was defined as blood pressure ≥140/90 mm Hg or use of blood pressure-lowering medication, smoking as current smoking. Blood pressure, body mass index (BMI), smoking status, hs-CRP, and cholesterol and lipid levels were determined as previously described [18, 19]. One subject was excluded because of incomplete clinical data. The study was approved by the Regional Ethical Review Board in Lund and was conducted in accordance with the Declaration of Helsinki. All participants gave written consent.
**B-mode ultrasound**

Analysis of common and bulb carotid intima–media thickness (IMT) was performed using an Acuson 128 CT system with a 7-MHz transducer as described previously [18].

**Isolation of mononuclear leukocytes**

Blood (15 mL) was collected in heparin tubes and layered on top of 15 mL Lymphoprep before centrifugation at 1350 g for 12 min at room temperature. The interface layer was carefully harvested and the cells were then washed twice with 0.9% NaCl (the first centrifugation was at 600 g and the second at 300 g, both for 10 min at room temperature). The cells were re-suspended in 1.7 mL autologous serum and 1.6 mL cold RPMI 1640 medium with 20% DMSO was added. The cells were frozen slowly by placing them in a Styrofoam box at -80°C overnight. Frozen mononuclear cells were stored at -140°C.

**Flow cytometry**

Cells were then thawed to room temperature within 2 min; subsequently 4 mL phosphate-buffered saline (PBS) containing 1% human serum (HS) at 37°C was added over 1–2 min and a further 4 mL was added over approximately 30 s. Gradual dilution of the DMSO in the frozen cells avoided cell damage by osmotic shock, and pre-warming the dilution medium helped to actively compensate for changes in osmotic pressure. Cells were centrifuged and suspended in RPMI 1640 supplemented with 10% HS (Invitrogen, Stockholm, Sweden), 1% sodium pyruvate, 1% HEPES, 1% penicillin/streptomycin, 1% L-glutamine and 0.1% β-mercaptoethanol (Gibco, Stockholm, Sweden) at a concentration of 2x10⁶ cells/mL. First, 4x10⁵ cells/well were incubated with PMA (10 ng), ionomycin (0.2 µg) and brefeldin A (1 µg, all from Sigma) for 4 hours at 37°C. Cells were washed and incubated with CD56-biotin, CD3-
PE/Cy7, CD4-PB and CD8-AF700 for 30 min at 4°C. The samples were then rinsed and incubated with strepavidin-PerCP for 20 min at 4°C. Cells were washed and incubated with Fix/Perm solution and permeabilization buffer and subsequently incubated with IFN-γ-PE (all antibodies were from Biolegend) for 30 min at 4°C. Samples were acquired on an ADP-Cyan flow cytometer (Beckman Coulter) and analysis was performed using FlowJo 7.5.5 (Treestar Inc.). Species-specific compensation beads (BD Biosciences, Stockholm, Sweden) coupled to the respective antibodies was used to compensate fluorescent signals and non-stained cells, and FMO (fluorescence-minus-one) control samples were used to set the negative population. CD14++CD16- monocytes, IFN-γ expressing Th1 cells, IL-4 expressing Th2 cells and CD4+FoxP3+ regulatory T cells were analyzed as previously reported [17, 20, 21]. Cells used for analysis of CD14++CD16- monocytes were not incubated with PMA/ionomycin and brefeldin A. Mononuclear leukocytes used in control experiments to study the effect of freezing and thawing were obtained from healthy volunteers. Plasma cytokine were determined by multiplexed immunoassay (MesoScale Discovery).

**Statistical analysis**

Measures of skewness and kurtosis were used to test for normal distribution of CD3+CD56+ T cell populations and clinical parameters. Differences between means of normally distributed continuous variables were assessed with independent sample *t* tests and differences in proportions between subjects with and without CVD events were assessed using the chi-squared test. Differences between means of non-normally distributed continuous variables were assessed using the non-parametric Mann–Whitney test. Spearman’s rank correlation coefficients were used to examine relationships among continuous variables. The relation between CD3+CD56+ NKT-like cell subsets (in tertiles) and incidence of first CVD events (coronary events or stroke) and cancer during
follow-up was assessed by Kaplan Meier survival curves and quantified by Log rank test. A Cox proportional hazards regression model was used to assess the hazard ratio (HR, and 95% confidence interval) of first CVD events in relation to tertiles of CD3$^+$CD56$^+$ T cell subsets. The model included age, sex; diabetes, blood pressure, lipid levels, smoking status and use of anti-diabetic or blood pressure-lowering medication. The proportionality of the hazards assumption was confirmed by visual inspection of log–negative log survival curves.

**Results**

Baseline clinical characteristics of the study cohort are shown in Table 1. Coronary cases were more often male, had diabetes and received blood pressure-lowering medication. They were also characterized by higher fasting blood glucose, LDL/HDL ratio and systolic blood pressure (table 1). Stroke cases were more often male, treated with anti-diabetic and blood pressure-lowering medication, and had higher fasting blood glucose levels and diastolic blood pressure. A total of 170 subjects developed cancer during follow-up. There were no significant differences in baseline clinical characteristics between subjects with or without incident cancer (data not shown). Eighteen subjects suffered from both an incident coronary event and cancer.

CD3$^+$CD56$^+$ cell subsets were analyzed in thawed samples of mononuclear leukocytes that had been frozen in autologous serum/DMSO at the MDC study baseline investigation in 1991–1994 and stored at -140°C. Flow cytometric analysis using the viability marker 7AAD in thawed CD45$^+$ leukocytes demonstrated that 95% of the cells were alive.
Analysis of CD3⁺CD56⁺ cell subsets

The following populations were analyzed; CD3⁺CD56⁺, CD3⁺CD4⁺CD56⁺ and CD3⁺CD8⁺CD56⁺ (see figure 1A for gating). Control experiments demonstrated that freezing and thawing did not influence the size of the CD3⁺CD56⁺ T cell population (figure 1B). However, it did result in a minor relative decrease of the CD3⁺CD8⁺ population suggesting that these cells may be more sensitive to freezing and thawing. The expression of IFN-γ in activated CD3⁺CD56⁺ T cell populations were assessed as the fraction of cells demonstrating positive staining as well as by the mean fluorescence intensity (MFI). The fraction of CD3⁺CD56⁺ T cells of all lymphocytes was 5.4±3.8%. The fraction of CD4⁺ and CD8⁺ in the total population of CD3⁺CD56⁺ T cells were 25.6±12.4% and 40.2±16.7% respectively, while the fractions of IFN-γ expressing CD3⁺CD4⁺CD56⁺ and CD3⁺CD8⁺CD56⁺ in the study cohort were 18.9±11.9% and 34.9±18.5%, respectively (expressed as % of all CD3⁺CD56⁺ T cells). Accordingly, 73.8% of the CD3⁺CD4⁺CD56⁺ and 86.8% of CD3⁺CD8⁺CD56⁺ T cells expressed IFN-γ when activated. CD3⁺CD56⁺ T cells have been referred to as NKT-like cells because they as classical invariant NKT cells express NK cell receptors [1]. To determine the relation between CD3⁺CD56⁺ T cells and classical NKT cells in the present study we used α-galactosylceramide-loaded CD1d tetramers. Only a minor fraction of the CD3⁺CD56⁺ T cells (1.3±0.2%) demonstrated positive tetramer staining and most of the α-galactosylceramide tetramer-positive cells were CD56 negative (figure 1C). CD56⁺, but not CD56⁻, cells expressed IFN-γ.

Associations of CD3⁺CD56⁺ T cells with other leukocyte populations and plasma cytokines
Generally CD3⁺CD56⁺ T cell populations and their expression of IFN-γ were strongly associated with the expression of IFN-γ (Th1 cells) and IL-4 (Th2 cells) in conventional (CD56⁻) T cells and more weakly with the presence of classical pro-inflammatory CD14++ CD16⁻ monocytes (table 2). There were also significant inverse correlations between the CD3⁺CD56⁺ T cell populations and the level of circulating Tregs (table 2). Notably, most associations were stronger for IFN-γ expressing CD3⁺CD56 T cells than for the corresponding total CD3⁺CD56⁺ T cell populations. A high number of CD3⁺CD56⁺ T cells in plasma were associated with increased levels of IL-8 but lower levels of IL-13 in plasma (table 3).

**CD3⁺CD56⁺ T cells and cancer**

There were no significant differences in baseline CD3⁺CD56⁺ T total cell number or fraction of CD3⁺CD56⁺ NKT-like cells between those with incident cancer and those that remained free of cancer during follow-up (table 4). Plotting CD3⁺CD56⁺ NKT-like cell tertiles into Kaplan-Meier curves also revealed no association with the incident cancer (figure 2 A and B and data not shown). When subjects with incident cancer were studied separately we found we found stronger associations between plasma IL-2 levels and both total CD4⁺CD56⁺ and IFN-γ expressing CD4⁺CD56⁺ NKT-like cells (-0.17, p<0.05 for both), but otherwise there were no major differences in these associations between those with and without incident cancer (data not shown).

**CD3⁺CD56⁺ T cells and cardiovascular disease**

There was no significant difference in CD3⁺CD56⁺ cell numbers or percent of the different NKT-like cell subsets between those with or without an incident cardiovascular event (table 4). However, Kaplan–Meier curves of event-free survival showed an increased incidence of coronary events in the group with the
lowest tertiles of CD4^+CD56^+IFN-γ^+ T cells as compared to the two highest tertiles (figure 2; log-rank test: P <0.05). This association remained significant when controlling for age, sex, smoking, BMI, hypertension, diabetes, the Th1/Th2 and the Th1/Treg cell ratio in a Cox proportional hazards regression model (hazard ratio 1.98, 95% C.I. 1.24-3.16), but not when LDL/HDL ratio was included in the model (hazard ratio: 1.61; 95% C.I. 0.92-2.82). There was no significant association between other CD3^+CD56^+ T cell tertiles and cardiovascular events. Moreover, there were no significant associations between CD3^+CD56^+ T cell populations (in total numbers or as percent) and intima-media thickness in the common carotid artery or the carotid bulb (data not shown). When subjects with incident coronary events were studied separately we found we found stronger associations between IL-5 and both CD4^+CD56^+ and CD8^+CD56^+ NKT-like cells (-0.23, p<0.05 and r=0.22, p<0.05, respectively), but otherwise there were no major differences between those with and without coronary events (data not shown).

**Association of CD3^+CD56^+ T cells with cardiovascular risk factors**

Correlations between CD3^+CD56^+ T cells and cardiovascular risk factor were generally weak but the general trend was that CD3^+CD56^+ T cells correlated positively with BMI and triglycerides and inversely with age and HDL (table 5).

**Discussion**

CD3^+CD56^+ T cells belong to a family of T cells that express NK markers but are distinct from classical invariant NKT cells by not being CD1d- restricted and are therefore referred to as NKT-like cells [1]. The present study tested the hypothesis that the level of circulating CD3^+CD56^+ NKT-like cells is associated with the risk for development of CVD and cancer. Since CD3^+CD56^+ T cells
have potent anti-tumor properties [6] that low frequencies of CD3^+CD56^+ T cells is associated with a more rapid progression of chronic lymphatic leukemia, lung and colon cancer [8, 9] and that ex vivo generated CD3^+CD56^+ T cells are used in clinical trials for treatment of cancer [10] it could be anticipated that in a general population high levels of these cells would be associated with a lower risk of cancer. However, we found no evidence for an association between circulating levels of CD3^+CD56^+ NKT-like cells and the incidence of cancer in the present study. The reason for the lack of association between cancer and CD3^+CD56^+ NKT-like cells in present study remains to be fully elucidated. However, it should be kept in mind that previous studies have investigated the relation between these cells and the clinical outcome of prevalent cancer rather the association with risk for cancer development. It is thus possible that CD3^+CD56^+ NKT-like cells are important for targeting existing cancer cells than in protecting against the development of new cancers.

With respect to the risk for CVD we found that subjects with low levels of IFN-γ expressing CD3^+CD4^+CD56^+ NKT-like cells had an increased incidence of coronary events, whereas no association were found with the incidence of stroke. The association between the fraction of IFN-γ expressing CD3^+CD4^+CD56^+ NKT-like cells and incidence of coronary events was independent of age, sex, smoking, BMI, hypertension and diabetes. However, the association lost statistical significance when also adjusting for HDL or the LDL/HDL ratio. Interestingly, the fraction of IFN-γ expressing CD3^+CD4^+CD56^+ T cells correlated significantly with HDL but not with LDL.

Atherosclerosis is a chronic inflammatory arterial disease responsible for the development of most cases of myocardial infarction and stroke [22]. The arterial inflammation in atherosclerosis is primarily driven by accumulation and oxidation of low density lipoprotein (LDL)-derived lipids. Evidence from
studies performed in animal models of atherosclerosis suggest that this inflammation can be modulated by adaptive immunity and that both protective and pathogenic immune responses are activated in response to arterial accumulation of oxidized LDL [23-26]. Immune responses mediated by CD4+ Th1, CD8+ T cells and NKT cells have been found to aggravate the disease process while regulatory T cells (Treg) are protective. The role of CD4+ Th2 and B cells is more complex and both protective and pathogenic subtypes have been described. Against this background there has been an increasing interest in exploring possible clinical associations between immune cells and risk for development of cardiovascular disease, but this has so far only been investigated in a limited number of prospective studies. It has been reported that low levels of CD4+ Th2 and Tregs as well as high levels of CD8+ T cells are associated with an increased risk for development of acute coronary events [20, 21, 27], while low levels of CD4+ Th2 and CD40+ B cells are associated with increased risk of stroke [28]. Autoimmune responses against epitopes in oxidized LDL are believed to aggravate atherosclerosis [24]. Since CD3+CD56+ NKT-like cells are increased in several autoimmune diseases [11-14] and as well as in patients with coronary disease [15] there is a possibility that they could be involved in the regulation of tolerance in atherosclerotic plaques. The correlation between CD3+CD56+ T cells and HDL suggest some type of interaction with lipoprotein lipids. Invariant NKT cells are a subset of T cells that share characteristics with both conventional T cells and natural killer cells and that are responsive to lipid antigens presented on the MHC class I-like molecule CD1 [29, 30]. CD3+CD56+ T cells have been referred to as NKT-like cells because both express the NK cell marker CD56 [1, 31]. We used α-galactosylceramide tetramers to identify CD1d-restricted NKT cells and found that most of these were not CD56 positive. Indeed, only about one percent of the CD3+CD56+ T cells were CD1d-restricted. However, it cannot be excluded that CD3+CD56+ NKT-like cells also respond to lipids presented on other CD1 molecules. The
association between low levels of IFN-γ expressing CD3⁺CD4⁺CD56⁺ T cells and increased risk of coronary events could argue for a protective role of these cells in cardiovascular disease. Most experimental studies have shown that inhibition of presentation of lipid antigens through the CD1d–NKT cell pathways results in a decreased development of atherosclerosis in hypercholesterolemic mice [32-35]. Again, this would argue against responsiveness to lipid antigens as the explanation to the association between CD3⁺CD56⁺ NKT-like cells and coronary events.

In the present study we found significant associations between circulating CD3⁺CD8⁺CD56⁺ NKT-like cells and plasma levels of the pro-inflammatory cytokines, such as IFN-γ and TNF-α, as well as the chemokine IL-8. There was also an inverse association between CD3⁺CD8⁺CD56⁺ T cells and the Th2-type cytokine IL-13. IFN-γ expressing CD3⁺CD56⁺ NKT-like cells correlated strongly with the presence of IFN-γ expressing Th1 cells and IL-4 expressing Th2 cells suggesting that they occur as part of a more general immune response. High levels of Th2 cells have previously been associated with a decreased risk for coronary events while no such association was found for Th1 cells [21]. It should also be noted that we observed a highly significant inverse correlation between IFN-γ expressing CD3⁺CD56⁺ NKT-like cells and Tregs in the present study. There is convincing evidence from experimental studies that Tregs have a protective function in atherosclerosis [36, 37] and we have recently shown that subjects with low levels of Tregs are at increased risk for coronary events [20]. Accordingly, it is unlikely that the association between high levels of IFN-γ expressing CD3⁺CD56⁺ NKT-like cells and a lower incidence of coronary events involves increased activation of Tregs. The observation that the association between IFN-γ expressing CD3⁺CD56⁺ NKT-like cells and incident coronary events remained significant when controlling for both the Th1/th2 as
well as the Th1/Treg ratio suggest that it is independent of interaction with other T cell types.

There are some limitations of the present study that should be considered. The studies were performed on cells that had been stored at -140 °C for several years. As compared to initiating new prospective studies this has the obvious advantage of allowing studies to be completed within a relatively short period of time but it remains to be clarified how representative thawed cells are of the original cell population. Although we did not observe any reduction of the fraction of CD3⁺CD56⁺ NKT-like cells in response to freezing and thawing of freshly isolated cells we did see a minor decrease in the fraction of CD3⁺CD8⁺ T cells which may have influenced the results of our study. However, around 30% of the CD3⁺CD56⁺ cells in the study cohort were CD4⁺ and CD8⁺ double negative. It cannot be excluded that this reflects a loss of CD4⁺ and CD8⁺ with extended freezing that we were unable to detect in our analyses of freshly isolated cells. Moreover, though this study was relatively large in comparison with many other clinical studies using characterization of leukocyte subtypes by flow cytometry it still is small in terms of power to predict clinical outcomes. Hence, it cannot be excluded that a larger study would have been able to detect other association between CD3⁺CD56⁺ NKT-like cells and cancer.

In conclusion, the present study could not confirm the hypothesis that low levels of CD3⁺CD56⁺ NKT-like cells are associated with a higher incidence cancer and a lower incidence of CVD. Contrarily, we found that low levels of IFN-γ expressing CD3⁺CD4⁺CD56⁺ NKT-like cells was associated with an increased incidence of coronary events. The findings provide the first clinical support for a role of CD3⁺CD4⁺CD56⁺ NKT-like cells in CVD.
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Disclosures
None
References

Figure 1

A

CD3+CD56

CD3+CD4+CD56

CD3+CD8+CD56

CD3+IFN-γ

CD3+CD4+CD56

CD3+IFN-γ

CD3+CD8+CD56

B

C

D

Unstim. PM/Ag

Unstim. PM/Ag

Unstim. PM/Ag
Figure 2

A

B

C

Follow-up period (in years) from screening to first detection or death

Follow-up period (in years) from screening to first cancer or death

Follow-up period (in years) from screening to first recurrence, event, or death
**Figure legends**

Figure 1. (A) Flow cytometric gating of the NKT populations CD3⁺CD56⁺, CD3⁺CD4⁺CD56⁺ and CD3⁺CD8⁺CD56⁺ from mononuclear cells (MNLs). The expression of IFN-γ in the different CD3⁺CD56⁺ cell populations were assessed as the fraction of cells demonstrating positive staining as well as by the mean fluorescence intensity (MFI). (B) Fraction of CD3⁺CD56⁺, CD4⁺ and CD8⁺ T cells in healthy controls before and after freezing and thawing. (C) Fraction of CD3⁺CD56⁺ and α-galactosylceramide-CD1d tetramer-positive T cells (out of all CD3⁺ T cells) in unstimulated and PMA/ionomycin stimulated cells from healthy controls. (D) Gating for α-galactosylceramide-CD1d tetramer-positive cells.

Figure 2. Kaplan-Meier curves of event-free survival of a cancer, coronary event or death. Tertiles of (A) CD4⁺CD56⁺ NKT-like cells, (B) CD8⁺CD56⁺ NKT-like cells and (C) lowest versus the two highest tertiles of CD4⁺CD56⁺IFN-γ⁺ NKT-like cells (expressed as % of all CD3⁺CD56⁺ cells for all) are shown.