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Association of Atherosclerosis With Expression of the LILRB1 Receptor By Human NK and T-Cells Supports the Infectious Burden Hypothesis

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Objective—The contribution of human cytomegalovirus (HCMV) to vascular disease may depend on features of the immune response not reflected by the detection of specific antibodies. Persistent HCMV infection in healthy blood donors has been associated with changes in the distribution of NK cell receptors (NKR). The putative relationship among HCMV infection, NKR distribution, subclinical atherosclerosis, and coronary heart disease was assessed.

Methods and Results—NKR expression was compared in acute myocardial infarction (AMI) patients ($n=70$) and a population-based control sample ($n=209$). The relationship between NKR expression and carotid intima-media thickness (CIMT) in controls ($n=149$) was also studied. HCMV infection was associated with higher proportions of NKG2C+ and LILRB1+ NK and T-cells. In contrast, only LILRB1+ NK and CD56+ T-cells were found to be increased in AMI patients, independent of age, sex, conventional vascular risk factors, and HCMV seropositivity. Remarkably, LILRB1 expression in NK and T-cells significantly correlated with CIMT in controls.

Conclusion—The association of overt and subclinical atherosclerotic disease with LILRB1+ NK and T-cells likely reflects a relationship between the immune challenge by infections and cardiovascular disease risk, without attributing a dominant role for HCMV. Our findings may lead to the identification of novel biomarkers of vascular disease. **(*Arterioscler Thromb Vasc Biol*. 2011;31:2314-2321.)**

Key Words: atherosclerosis ■ leukocytes ■ risk factors ■ viruses

Inflammation is a hallmark in the pathogenesis of atherosclerosis, which causes myocardial infarction and stroke.[1,2] Endothelial dysfunction is considered an initial event in the development of atherosclerotic plaques, as it promotes the migration of leukocytes and monocytes into the vessel wall, where macrophage interactions with T-cells are believed to play an important pathogenic role.[3] Beyond the influence of conventional risk factors (ie, smoking, hypertension, diabetes mellitus, hypercholesterolemia), infections have been also related to atherosclerosis.[4–6] Yet, the issue has remained controversial owing to the fact that the circumstantial evidence does not fulfill conventional criteria for causality, and the mechanisms whereby microbial pathogens might contribute to atherogenesis are uncertain. In fact, different bacteria and viruses have been associated with vascular disease, mainly based on seroepidemiological studies that provide only partial information on the host–pathogen relationship.[7,8] Moreover, infectious agents have been only occasionally isolated from vascular lesions,[9] and clinical trials have failed to show any beneficial effect of antibiotic therapy on myocardial infarction recurrence or long-term complications in patients with chronic coronary disease.[10,11] The infectious burden hypothesis reconciled to some extent apparently contradictory studies, proposing that different microbial agents contribute to the risk of vascular disease in a cumulative manner; yet, the search for suitable biomarkers to further validate the infectious burden hypothesis is warranted.[12,13]

Human cytomegalovirus (HCMV) is believed to be involved in the development of atherosclerosis, mainly based on 3 lines of evidence: (1) the epidemiological observation of a higher frequency of HCMV seropositivity in patients with atherosclerotic diseases as compared with healthy controls, not confirmed by all studies[14,15]; (2) observations linking

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HCMV to vascular lesions in chronic graft rejection and coronary restenosis postangioplasty[16,17]; and (3) the ability of murine cytomegalovirus infection to accelerate the development of vascular lesions in Apo-$E^{-/-}$ mice, an effect also promoted by the inactivated virus, thus supporting an indirect contribution to the inflammatory process.[18,19]

HCMV infection is highly prevalent and the virus remains in a lifelong latent state in healthy individuals, occasionally undergoing subclinical reactivations.[20] Beyond its pathogenic role in immunocompromised patients and congenital infection, HCMV seropositivity has been proposed as being linked to an accelerated immunosenescence and a shorter lifespan.[21,22] It is conceivable that the putative role of HCMV in the pathogenesis of atherosclerosis may ultimately depend on features of the complex host-pathogen interaction not reflected by the simple detection of circulating specific antibodies.[23–25]

In this regard, it has been shown that HCMV infection may alter to a variable extent the distribution of NK cell receptors (NKR). Increased proportions of NK and T-cell subsets expressing CD94/NKG2C, an activating lectin-like NKR specific for the HLA-E class I molecule,[26,27] were associated with a positive serology for HCMV[28–31]; moreover, NKG2C+ NK cells expanded in vitro in response to HCMV-infected fibroblasts.[28,32]

In addition, increased proportions of LILRB1+ NK and T-cells[28] were also detected in HCMV+ individuals. LILRB1 (ILT2, LIR-1, CD85j) is an inhibitory receptor expressed by different leukocyte lineages, which specifically interacts with HLA class I molecules and the UL18 HCMV glycoprotein[33,34] regulating cell activation. LILRB1 expression has been associated with late differentiation stages of T lymphocytes specific for different microbial pathogens.[35–37]

In the present study, we addressed whether the impact of HCMV infection on the NKR distribution might reflect its putative role in the pathogenesis of atherosclerosis. To challenge this hypothesis, a population-based case-control study was designed comparing the expression of NKG2C and LILRB1 in NK and T-cells from patients studied within 72 hours after acute myocardial infarction (AMI) and from control individuals without clinical evidence of cardiovascular disease. In a subsample of the latter, the relationship between NKR expression and carotid intima-media thickness (CIMT) was also assessed.

Methods

For a supplemental methods description, please access http://atvb.ahajournals.org.

Design and Subjects

Two different designs were used to test our hypothesis:(1) A case-control study was carried out to assess the association between NKR expression and AMI. Cases were 70 patients (aged 34 to 87 years) with confirmed AMI (Hospital Trueta, Girona and Hospital del Mar, Barcelona, Spain). AMI cases related to invasive procedures in which embolic events could play a role were excluded. Controls were contemporarily participants in a population-based cohort study (REGICOR-HERMES, Girona, Spain)[38] undertaken in the same area. The main aim of this cohort study was to determine the prevalence of classical and emergent cardiovascular risk factors and their predictive value. To control for differences in sex and age, we also carried out an age- and sex-matched case-control study in a subsample including 62 cases and 124 controls.(2) A cross-sectional study was designed to assess the association between NKR expression and CIMT in a group of participants of the population-based cohort study free of clinical disease ($n=149$).

In cases, blood samples were obtained within the first 72 hours after symptom onset ($n=70$) and also 6 months later to the acute event ($n=53$). Written informed consent was obtained from every donor, and the study protocol was approved by the local Ethics Committee (CEIC, Parc de Salut Mar).

Antibodies and Reagents

Anti-NKG2C–PE monoclonal antibody (mAb) was from R&D Systems, Inc Z199 (anti-NKG2A) mAb was kindly provided by Dr A. Moretta (University of Genova). Anti-LILRB1-FITC, CD3-PerCP, and CD56-APC were from BD Biosciences Pharmingen (San Jose, CA). Erythrocytes were lysed using FACS lysis buffer (Becton Dickinson). An ELISA kit (Bioelisa CMV Color; Biokit, Barcelona, Spain) was used to determine circulating antibodies against HCMV. High-sensitivity C-reactive protein (hs-CRP) was measured by immunoturbidimetry (ABX-Horiba Diagnostics, Irvine, CA).

Immunofluorescence and Flow Cytometry Analysis

The expression of NKG2A, NKG2C, and LILRB1 was analyzed by flow cytometry in fresh peripheral blood samples.[29] For multicolor staining whole blood samples were incubated with anti-NKG2A–FITC, anti-NKG2C–PE, anti-CD56–APC, and anti-CD3–PerCP or with anti-LILRB1, anti-CD3, and anti-CD56. After washing, erythrocytes were lysed. It is of note that in our first original report the analysis was restricted to the $NKG2C^{bright}$ cell subset as indicated,[28] whereas total NKG2C+ lymphocytes were considered in the present study.

Carotid Artery Ultrasound

B-mode ultrasound imaging of the carotid arterial walls was used to assess intima-media thickness according to standardized and validated imaging and image analysis protocols. These protocols have been described in detail elsewhere.[39] A specifically designed REGICOR scan application protocol was developed for the ultrasound equipment to ensure standardization throughout the study. Bilaterally, from a single latero-lateral transducer angle, the far walls of the common carotid were imaged by B-mode ultrasound.

Statistical Analysis

Normality plots were used to assess whether a continuous variable followed a normal distribution or not. Continuous normal distributed variables were summarized as means and standard deviations; continuous non-normal distributed variables were summarized as medians and first and third quartiles; categorical variables were presented as absolute frequencies and proportions. Student *t* test was used to compare means for normal distributed variables and Mann-Whitney U test was used to compare medians for continuous non-normal distributed variables. Chi-squared test or exact Fisher test was used as appropriate to compare proportions. Spearman correlation was used to assess the association between continuous variables.

To estimate adjusted means, multivariate linear regression was fitted. A logarithmic transformation was done if the response variable distribution departed from normality.

For age- and sex-matched case-control analysis, an algorithm was applied with the following criteria: every case was matched to 2 controls of the same sex and with a similar age (±5 years). Statistical analysis was done with R software, version 2.10.1. Statistical significance was set up at probability values <0.05.

Results

The expression of NKR was analyzed by flow cytometry in fresh blood samples from AMI patients ($N=70$) and controls ($N=209$). NK cells (CD3− CD56+), T lymphocytes

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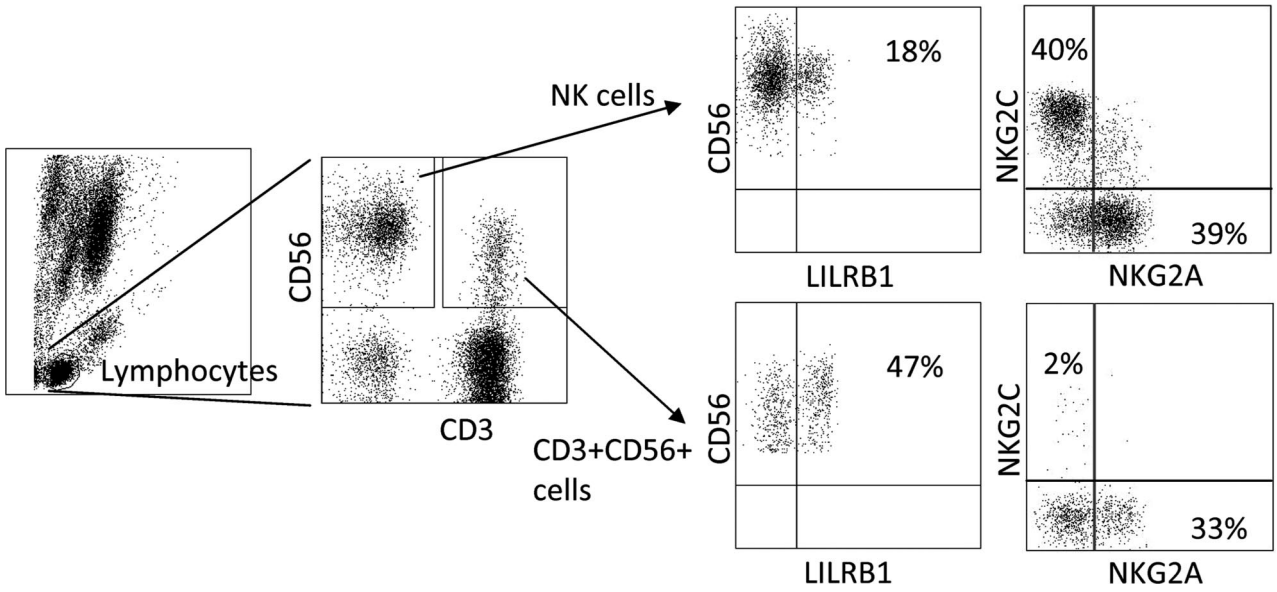


Figure. Analysis of NKG2A, NKG2C, and LILRB1 expression. Whole blood from a healthy donor was stained with anti-LILRB1, anti-CD3, and CD56 mAbs. Samples were analyzed by flow cytometry and the proportions of LILRB1+ cells were calculated gating NK cells (CD3+ CD56−) as well as the CD3+ CD56+ T-cell subset. Alternatively, whole blood was stained with anti-NKG2C, anti-NKG2A, anti-CD3, and anti-CD56 mAbs. Numbers correspond to the percentages of positive cells.

(CD3+), and T-cell subsets defined by CD56 expression were gated (Figure). Conventional risk factors for cardiovascular disease, HCMV seroprevalence, and hs-CRP levels in cases and controls are displayed in Table 1. Considering the demographical differences observed, a subsample of age- and sex-matched cases ($N=62$) and controls ($N=124$) was separately analyzed (Table 1).

Association of NKG2C and LILRB1 Expression With HCMV Seropositivity

As shown in Table 1, the frequencies of HCMV seropositive age- and sex-matched AMI patients and controls were significantly different (88.5% versus 75.8%; $P=0.042$). The influence of HCMV infection on the immunophenotype was analyzed by pooling data from all cases and controls, adjusting for age, sex, and cardiovascular risk factors, as well as for the incidence of AMI (Table 2). In agreement with previous reports,[28–31] the proportions of NKG2C+ NK cells and LILRB1+ NK and T-cells were higher in samples from HCMV+ individuals that, conversely, contained lower proportions of NK cells bearing the NKG2A inhibitory receptor. LILRB1 and NKG2C expression were increased in both the CD56− and CD56+ T-cell subsets, whereas differences in the distribution of NKG2A+ cells were only significant in the CD3+ CD56+ population. This minor T-cell subset was also increased in HCMV+ subjects, and no differences in total NK and T-cells were noticed. The association of these immunophenotypic features with a positive HCMV serology was independent of age, sex, cardiovascular risk factors, and AMI, as potentially confounding factors. Similar results were obtained when the analysis was restricted to the subsample of age- and sex-matched cases and controls (Supplemental

Table 1. Cardiovascular Risk Factors In Acute Myocardial Infarction Cases and Controls

	Total Controls N=209	Total Cases N=70	*P*-Value	Matched Controls§ N=124	Matched Cases§ N=62	*P*-Value
Age (years)*	57.0 (11.4)	62.9 (12.3)	**<0.001**	60.5 (11.0)	60.7 (11.1)	0.910
Sex (% female)†	63 (30.1)	11 (15.7)	**0.018**	18 (14.5%)	9 (14.5%)	>0.999
Active smoker or ex <1 year (%)†	50 (24.3)	29 (41.4)	**0.006**	31 (25.2%)	28 (45.2%)	**0.006**
BMI (kg/m^2)*	27.8 (3.98)	26.7 (4.18)	**0.048**	27.7 (3.76)	26.8 (4.20)	0.140
Informed of hypertension†	78 (37.3)	47 (70.1)	**<0.001**	50 (40.3%)	40 (67.8%)	**0.001**
Informed of high blood glucose (%)†	25 (12.0)	23 (33.8)	**<0.001**	17 (13.7%)	17 (28.3%)	**0.017**
Informed of high cholesterol (%)†	77 (36.8)	35 (52.2)	**0.026**	50 (40.3%)	30 (50.8%)	0.180
hs-CRP (mg/dL)‡	0.15 (0.05–0.34)	2.12 (1.06–5.23)	**<0.001**	0.15 (0.06–0.33)	1.89 (0.98–4.46)	**<0.001**
HCMV serology (HCMV-positive %)†	167 (79.9)	61 (88.4)	**0.111**	94 (75.8%)	54 (88.5%)	**0.042**

*Data are expressed as mean (SD). Statistical analysis according to the Student *t* test.
†Frequency (%). Statistical analysis according to the χ^2 test or Fisher exact test as appropriate.
‡Data are expressed as median (1st–3rd quartiles). Statistical analysis according to the Mann-Whitney test.
§Sex and age (±5 years) matched subsample of cases and controls.
In bold, significant *P*-values are marked.

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Table 2. NK Cell Receptors (NKR) Expression Analysis Comparing Human Cytomegalovirus (HCMV) Positive and Negative Individuals Adjusted By Sex, Age, Cardiovascular Risk Factors,* and Acute Myocardial Infarction

Receptor†	Subset†	HCMV-Negative‡ N=50	HCMV-Positive‡ N=228	*P*-Value§
	$CD3^+$ $CD56^-$	72.7 [69.2; 76.3]	73.2 [71.7; 74.8]	0.806
	$CD3^+$ $CD56^+$	1.03 [0.76; 1.39]	2.66 [2.34; 3.03]	**<0.001**
	NK	8.03 [6.37; 10.1]	7.09 [6.41; 7.83]	0.333
NKG2A	$CD3^+$	0.71 [0.50; 1.01]	1.01 [0.86; 1.19]	0.080
	$CD3^+$ $CD56^-$	2.32 [1.85; 2.92]	2.04 [1.83; 2.27]	0.318
	$CD3^+$ $CD56^+$	12.7 [9.06; 17.9]	7.67 [6.56; 8.97]	**0.009**
	NK	36.6 [32.7; 40.4]	31.4 [29.6; 33.2]	**0.018**
NKG2C	$CD3^+$	1.25 [0.92; 1.70]	1.80 [1.56; 2.07]	**0.037**
	$CD3^+$ $CD56^-$	0.29 [0.21; 0.40]	0.49 [0.42; 0.57]	**0.004**
	$CD3^+$ $CD56^+$	2.40 [1.57; 3.67]	5.26 [4.35; 6.36]	**0.001**
	NK	3.98 [2.88; 5.51]	7.54 [6.48; 8.77]	**0.001**
LILRB1	$CD3^+$	2.16 [1.65; 2.83]	5.28 [4.65; 5.98]	**<0.001**
	$CD3^+$ $CD56^-$	1.69 [1.30; 2.19]	4.10 [3.63; 4.63]	**<0.001**
	$CD3^+$ $CD56^+$	10.3 [7.99; 13.3]	20.7 [18.4; 23.2]	**<0.001**
	NK	12.6 [10.2; 15.7]	17.5 [15.8; 19.3]	**0.009**

*Risk factors: smoking, BMI, informed of hypertension, informed of high glucose, informed of high cholesterol.

†Data are expressed as the proportions (%) of cells expressing every receptor within the defined lymphocyte subsets.

‡Data are expressed as mean [IC 95%].

§Statistical analysis according to the Mann-Whitney test.

In bold, significant *P*-values are marked.

Table). Regression analysis did not reveal any significant correlation between NKG2C+ NK cells and LILRB1+ NK cells (r=0.059), LILRB1+ T lymphocytes (r=0.024), nor LILRB1+ CD56+ T-cells (r=0.007), supporting a dissociated expression of both receptors.

Increased Proportions of LILRB1+ NK and T-Cells in AMI Patients

When NKR distribution was compared in age- and sex-matched cases and controls, the proportions of LILRB1+ NK and T-cells, as well as the CD3+ CD56+ subset, were significantly increased in AMI patients; moreover, the relative numbers of total NK and T-cells also differed (Table 3). Remarkably, NKG2C and NKG2A expression was undistinguishable between cases and controls (Table 3), in contrast to the effect of HCMV infection on these markers (Table 2). Similar results were obtained when total cases and controls were analyzed (data not shown). The increased proportions in AMI patients of LILRB1+ NK cells, LILRB1+ CD56+ T-cells, as well as total CD3+ CD56+ cells remained consistent after adjusting for cardiovascular risk factors, HCMV seropositivity (Table 3), and therapy with statins (24.3% cases and 13% controls, P=0.025). NKR expression was also compared in cases and controls according to their HCMV serological status. Among HCMV+ individuals, the proportions of CD3+ CD56+, LILRB1+ NK and CD56+ T-cells were increased in AMI patients, whereas no significant differences were found between HCMV seronegative cases and controls (data not shown). Yet, the statistical power of this latter analysis was limited due to the few HCMV-negative cases (n=7).

A low but significant correlation between the proportions of LILRB1+ NK and T-cells (r=0.2; P<0.001) was noticed. Moreover, hs-CRP levels were elevated in AMI cases (Tables 1 and 3) correlating with LILRB1+ NK (r=0.30, P=0.014) and CD56+ T-cells (r=0.30, P=0.016) (n=69).

Relationship of CIMT With Increased Proportions of LILRB1+ NK and T-Cells

When samples obtained from cases (n=53) >6 months after the acute event were analyzed, differences in LILRB1 expression with controls persisted (data not shown), indirectly suggesting that the immunophenotypic profile was not attributable to the acute phase of AMI, and might predate the development of the disease. To address this issue, we studied the putative relationship between NKR expression and subclinical atherosclerosis, measuring CIMT in control individuals (n=149) (Table 4). Remarkably, the proportions of LILRB1+ NK and T-cells directly correlated with CIMT. After adjusting for age, sex, cardiovascular risk factors, and HCMV serology (Table 4), the correlation with CIMT remained significant for LILRB1 expression in NK and CD56− T-cells. In contrast, the proportions of LILRB1+ CD56+ T lymphocytes and of total CD3+ CD56+ cells did not correlate with CIMT, at variance with the observations in AMI. Altogether the data further supported that LILRB1 expression by NK and T-cells may be independently associated with atherosclerosis.

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Table 3. Descriptive Analysis of NK Cell Receptors (NKR) Expression Comparing Age and Sex-Matched Acute Myocardial Infarction (AMI) Cases and Controls

	Subset*	Control N=124	Case N=62†	*P*-Value	Adjusted *P*-Value‡	Adjusted *P*-Value¶
	$CD3^+$ $CD56^-$	71.4 (65.3–79.3)	78.7 (68.7–83.3)	**0.014**	**0.031**	**0.036**
	$CD3^+$ $CD56^+$	1.86 (1.03–3.06)	3.31 (1.56–5.98)	**0.003**	**0.002**	**0.010**
	NK	9.54 (4.34–13.0)	5.79 (3.65–11.7)	**0.047**	0.102	0.056
NKG2A*‖	$CD3^+$	1.27 (0.38–2.72)	0.75 (0.34–2.59)	0.257	0.688	0.186
	$CD3^+$ $CD56^-$	2.12 (1.14–3.53)	2.08 (1.33–3.58)	0.832	0.763	0.857
	$CD3^+$ $CD56^+$	8.61 (3.51–18.9)	8.97 (4.16–20.5)	0.753	0.984	0.748
	NK	30.2 (23.6–41.4)	31.7 (22.6–42.3)	0.834	0.736	0.701
NKG2C*‖	$CD3^+$	1.63 (0.77–2.89)	1.57 (0.76–3.58)	0.825	0.967	0.633
	$CD3^+$ $CD56^-$	0.48 (0.19–1.09)	0.42 (0.15–0.82)	0.447	0.730	0.222
	$CD3^+$ $CD56^+$	3.92 (1.35–13.2)	4.97 (1.49–15.4)	0.375	0.478	0.777
	NK	6.19 (3.55–12.1)	7.45 (4.06–15.1)	0.226	0.620	0.434
LILRB1*‖	$CD3^+$	4.52 (2.43–8.76)	5.19 (3.11–10.1)	0.223	0.184	0.403
	$CD3^+$ $CD56^-$	3.39 (1.61–6.71)	3.90 (2.78–6.61)	0.164	0.138	0.267
	$CD3^+$ $CD56^+$	18.5 (8.88–35.9)	30.3 (15.3–56.8)	**0.003**	**0.023**	**0.030**
	NK	17.0 (10.4–28.9)	24.4 (13.1–41.0)	**0.003**	**0.014**	**0.004**
hs-CRP (mg/dl)‖		0.15 (0.06–0.33)	1.89 (0.98–4.46)	**<0.001**	**<0.001**	**<0.001**
HCMV serology (HCMV-positive %)§		94 (75.8)	54 (88.5)	**0.042**	**0.038**	. . .

*Data are expressed as the proportions (%) of cells expressing every receptor within the indicated lymphocyte subsets.
†Eight cases remaining unmatched were excluded.
‡Adjusted by informed of HTA, informed of high glucose and smoking.
§Frequency (%). Statistical analysis according to the χ^2 test or Fisher exact test as appropriate.
‖Data are expressed as median (1^{st}–3^{rd} quartiles). Statistical analysis according to the Mann-Whitney test.
¶Adjusted by HCMV serology.
Hs-CRP indicates high-sensitivity C-reactive protein.

Hs-CRP concentrations have been previously related not only with acute coronary disease but also with subclinical atherosclerosis. In our analysis, hs-CRP levels significantly correlated with CIMT in controls ($n=149$; $r=0.252$, $P=0.002$), but not with the proportions of LILRB1+ lymphocytes, in contrast to the observations in AMI.

LILRB1+ NK and T-Cells Display A Late Differentiation Phenotype

Surface LILRB1 expression has been related to late stages of T-cell differentiation.[35] Multicolor flow cytometry analysis performed in a limited number of donors confirmed that LILRB1 was coexpressed with CD45RA mainly in CD8+ T-cells (Supplemental Figure IA and IB). In the same line, LILRB1+ NK cells were predominantly found among the CD27-negative subset[40] (Supplemental Figure IC). Altogether, the data point out that LILRB1+ NK and T-cells associated with atherosclerosis display a phenotypic profile corresponding to late differentiation stages in both lineages.

Discussion

Despite a wide number of studies addressing the role of HCMV in the pathogenesis of atherosclerosis and vascular disease, the issue remains open. This may be explained by a high prevalence of the persistent infection, an overlapping involvement of other microbial pathogens, and the possibility that its contribution may be indirect.[24] Moreover, the detection of HCMV-specific antibodies used in conventional studies is not informative on the complex host–pathogen relationship, which may be a determinant of cardiovascular risk at the individual level. On that basis, we addressed whether the influence of HCMV infection on the distribution of NKG2C and LILRB1 NKR might reflect its putative contribution to the pathogenesis of atherosclerosis.

Our results supported that increased proportions of circulating LILRB1+ NK and T-cells were associated with AMI, independently of age, sex, conventional cardiovascular risk factors, and HCMV infection. Remarkably, these immunophenotypic features appeared as well related to carotid plaque thickness, a measurement of preclinical atherosclerotic disease, in control participants, thereby suggesting that such profile might contribute to predict future cardiovascular disease development.

Though increased LILRB1+ NK cells were associated with both AMI and CIMT, some differences were noticed between both settings. In fact, CIMT correlated with the proportions of LILRB1+ CD56− T-cells, whereas AMI appeared associated with LILRB1 expression in CD56+ T-cells. Moreover, increased proportions of total T lymphocytes and CD56+ T-cells were associated with AMI but not with CIMT. The CD56+ T-cell subset includes cytotoxic CD8+ and some CD4+ cells, often bearing NK cell associated molecules.[41] In the same line, LILRB1 expression correlated with elevated hs-CRP levels in AMI patients but not in controls. The relationship of the different immunophe-

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Table 4. Correlation Between NKR Expression and CIMT in Control Subjects

Receptor*	Subset*	Correlation† CCA IMT‡§	Adjusted Correlation‖ CCA IMT‡§	Adjusted Correlation¶ CCA IMT‡§
	$CD3^+$ $CD56^-$	**−0.284 (0.005)**	−0.165 (0.132)	−0.147 (0.183)
	$CD3^+$ $CD56^+$	−0.163 (0.113)	−0.055 (0.619)	−0.088 (0.424)
	NK	**0.207 (0.043)**	0.048 (0.661)	0.040 (0.715)
NKG2A	$CD3^+$	0.056 (0.516)	−0.035 (0.695)	−0.036 (0.685)
	$CD3^+$ $CD56^-$	**−0.241 (0.004)**	−0.103 (0.246)	−0.086 (0.339)
	$CD3^+$ $CD56^+$	**−0.189 (0.026)**	−0.134 (0.130)	−0.104 (0.244)
	NK	0.027 (0.754)	−0.064 (0.473)	−0.040 (0.650)
NKG2C	$CD3^+$	**−0.236 (0.004)**	−0.102 (0.241)	−0.110 (0.206)
	$CD3^+$ $CD56^-$	−0.014 (0.866)	−0.021 (0.813)	−0.051 (0.564)
	$CD3^+$ $CD56^+$	−0.054 (0.519)	−0.001 (0.994)	−0.032 (0.717)
	NK	0.057 (0.497)	−0.040 (0.645)	−0.079 (0.364)
LILRB1	$CD3^+$	**0.229 (0.006)**	**0.234 (0.006)**	**0.194 (0.025)**
	$CD3^+$ $CD56^-$	**0.203 (0.014)**	**0.278 (0.001)**	**0.242 (0.005)**
	$CD3^+$ $CD56^+$	**0.273 (0.001)**	0.094 (0.278)	0.044 (0.614)
	NK	**0.257 (0.002)**	**0.227 (0.008)**	**0.198 (0.022)**

*The proportions of cells expressing every receptor within the indicated lymphocyte subsets were considered as markers (predictors).

†Numbers correspond to the Spearman correlation (*P*-value) between CCA IMT and the percentage of receptor expressing cells.

‡Right and left common carotid artery (CCA) IMT mean.

§n=149.

‖Partial correlation (*P*-value) of the markers with CIMT, adjusting for age, sex, systolic blood pressure, LDL/HDL ratio, smoking, blood pressure treatment, and history of diabetes.

¶Partial correlation (*P*-value) of the markers on CIMT, adjusting for age, sex, systolic blood pressure, LDL/HDL ratio, smoking, blood pressure treatment, history of diabetes, and cytomegalovirus serology.

In bold, significant *P*-values are marked.

notypic features with the pathogenesis of atheroma formation versus acute coronary disease deserve further attention; the possibility that some of the observed changes may simply encompass the development of vascular disease is not ruled out. Despite the persistence along time of the immunophenotypic features associated to AMI, the possibility that they might be secondary to the ischemic event could not be formally ruled out. Further studies in patients with stable CHD might contribute to clarify the issue. Moreover, analyzing NKR expression in CD4 and CD8 T-cell subsets, as well as the absolute numbers of the different cell populations, would be of interest to refine the picture.

In agreement with previous studies,[15] a higher frequency of HCMV+ samples was detected among AMI cases as compared to age- and sex-matched controls. Nevertheless, the associations of AMI and CIMT with increased LILRB1+ cells appeared independent of HCMV seropositivity. Moreover, AMI and CIMT were unrelated to the expression levels of NKG2C, a marker that was confirmed to be linked to this viral infection, in line with previous reports.[28–31] The dissociated expression of both NKR may be explained by the ability of other microbial pathogens to expand LILRB1+ cells.[36,37] On the other hand, HCMV infection may promote an increase of LILRB1+ cells without expanding in parallel NKG2C+ NK cells. In fact, a wide variability in the proportions of this subset was found among HCMV+ individuals[28] without a significant correlation with LILRB1+ cells. Studies in immunodeficiencies[29,30,42] suggest that the magnitude of the NKG2C+ NK cell expansion may be inversely related to the efficiency of the T-cell–mediated response to the virus. In summary, despite that our results do not support a dominant role for HCMV, its contribution to the pathogenesis of atherosclerosis is not ruled out and might be related to the quality of the T-cell response to the infection, rather than to its influence on the distribution of the NKG2C+ NK cell subset. In this regard, the role of NK cells in atherosclerosis remains uncertain[1,43] but T lymphocyte subsets appear to play a complex role in the development of vascular lesions,[1–3] and NKR+ T-cells have been identified in atheroma plaques.[44]

The observation that NK and T-cells use the same LILRB1 promoter region[45] suggests that its expression may be controlled by common signals in both lineages. LILRB1 is mainly detected in differentiated T-cells specific for different pathogens, which display the CD28− CCR7low and CD45RA+ phenotype, and may coexpress CD56 and NKR (ie, CD94/NKG2, KIR).[35–37] Circulating LILRB1+ T-cells have been shown to be predominantly CD8+ but a subset of cytotoxic CD4+ CD56+ LILRB1+ cells expressing different NKR has been also identified in normal blood donors.[46] Our studies in a limited number of individuals confirmed this immunophenotypic profile. It is of note that the LILRB1 inhibitory receptor has been reported to dampen the response of terminally differentiated virus-specific T-cells,[37] whose

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expansion has been associated with immunosenescence.[22] Interestingly, Spyridopoulos et al recently reported a marked reduction of telomere length in CD8+ T lymphocytes from coronary heart disease (CHD) patients that correlated with cardiac dysfunction and was particularly evident in HCMV seropositive individuals, suggesting as well a link with immunosenescence.[47]

Similarly to T and B lymphocytes, activated NK cells may undergo clonal expansions and experience differentiation events that modify their phenotype and survival.[48,49] In this regard, LILRB1 is displayed by a variable fraction of CD56dim NK cells, being virtually undetectable in the CD56bright subset, reported to bear longer telomeres.[50] In the same line, CD27 expression has been related to early stages of mature NK cell differentiation,[40] and most LILRB1+ cells were predominantly found among the CD27-negative population.

Altogether, these observations support that the increase of LILRB1+ NK and T-cells associated with atherosclerosis correspond to an accumulation of differentiated lymphocytes, likely reflecting the pressure of persistent/recurrent infections. Though weak, a correlation between LILRB1+ NK and T-cells suggests that both cell lineages might be responding to common challenges, presumably involving intracellular pathogens. In this context, the relationship of atherosclerosis with other late differentiation markers in NK and T-cells should be considered. On the other hand, an association of hs-CRP levels with AMI and CIMT was observed, confirming previous reports.[13,51] It is conceivable that an increased production of the acute phase protein might reflect as well the influence of persistent infections.

Whether LILRB1+ NK and T-cells participate in the pathogenesis of vascular lesions or merely constitute an indirect marker of the influence exerted by infections deserves further attention. Exploring the dominant antigen specificities of LILRB1+ and CD56+ T-cells in individuals with atherosclerosis might eventually provide further insights on the nature of infectious agents contributing to the development of vascular lesions. As previously discussed, it is noteworthy that the lack of association of AMI and CIMT with an expansion of the NKG2C+ NK cell subset does not dismiss a significant contribution of HCMV to the role of the infectious burden in the pathogenesis of atherosclerosis.

Some limitations of the present study warrant attention. From a methodological standpoint, the analysis of leukocyte surface markers by flow cytometry requires the use of fresh or cryopreserved cells, as well as a precise standardization of technical protocols. Moreover, our observations are based on the analysis of a small population sample from a geographical area characterized by a low incidence of cardiovascular disease,[38,52] and the study design does not allow the establishment of causality. Finally, it cannot be ascertained whether the immunophenotypic changes associated to AMI indeed predate the development of the acute episode; studies in stable CHD would be helpful to approach this issue.

In summary, our findings may have clinical implications, opening perspectives for research on the role of infections in atherosclerosis and potentially leading to the identification of novel biomarkers of vascular disease development, irrespective of classical risk factors.

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Disclosures

An active license agreement for the commercialization of a LILRB1-specific monoclonal antibody (clone HP-F1), generated by Miguel López-Botet, has been established by the Pompeu Fabra University with eBioscience.

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SUPPLEMENT MATERIAL

Detailed Methods

Design and subjects

Two different designs were used to test our hypothesis:

a) A case-control study was carried out to assess the association between NKR expression and AMI. Cases were 70 patients (aged 34 to 87 years) with confirmed AMI (Hospital Trueta, Girona and Hospital del Mar, Barcelona, Spain). Controls were contemporarily participants in a population-based cohort study (REGICOR-HERMES, Girona, Spain)[1] undertaken in the same area (3 controls per case). To control for differences in sex and age we also carried out an age- and sex-matched case-control study in a subsample including 62 cases and 124 controls.

b) A cross-sectional study was designed to assess the association between NKR expression and carotid intima media thickness (CIMT) in a group of participants of the population-based cohort study free of clinical disease (n=149).

In cases, blood samples were obtained within the first 72 h after symptom onset (n=70) and also 6 months later to the acute event (n=53). All the biological samples were coded, shipped at 4ºC to a central laboratory and analysed within a period of 48h. Waist perimeter was measured at the middle point between the last rib and iliac crest. Height and weight were measured with calibrated instruments and subjects in underwear. Body mass index (BMI) was calculated as weight (kg)/ height $(m)^2$. Written informed consent was obtained from every donor, and the study protocol was approved by the local Ethics Committee (CEIC, Parc de Salut Mar).

Antibodies and reagents

Anti–NKG2C-PE monoclonal antibody (mAb) was from R&D Systems, Inc. Z199 (anti-NKG2A) mAb was kindly provided by Dr. A. Moretta (University of Genova). Z199 mAb was conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO). Anti-LILRB1-FITC, CD3-PerCP, CD56-APC, CCR7-PE-Cy7, CD8-PE and CD4-PE were from BD Biosciences Pharmingen (San Jose, CA). Anti-CD45RA-APC was from Immunotools (Friesoythe, Germany). Anti-CD27 mAb (clone 143-14) was kindly provided by Dr. R. Vilella

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(Hospital Clinic, Barcelona), and the HP-F1 anti-LILRB1 mAb was generated in our laboratory [2]. Indirect immunofluorescence analysis was carried out with FITC-tagged or PE-tagged $F(ab')_2$ rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). In whole blood samples, erythrocytes were lysed using FACS lysis buffer (Becton Dickinson). A commercially available ELISA kit (Bioelisa CMV Colour; Biokit, Barcelona, Spain) was used to determine circulating antibodies against HCMV. High-sensitivity C-reactive protein (hs-CRP) was measured by immunoturbidimetry (ABX-Horiba Diagnostics, Irvine, CA) in an autoanalyser PENTRA-400 (ABX-Horiba Diagnostics, Irvine, CA).

Immunofluorescence and flow cytometry analysis

The expression of NKG2A, NKG2C and LILRB1 was analysed by flow cytometry in fresh peripheral blood samples, obtained by venous puncture in EDTA tubes. For multicolor staining the following procedures were used. Protocol 1: whole blood samples were incubated with anti–NKG2A-FITC; subsequently, samples were incubated with anti–NKG2C-PE, anti–CD56-APC, and anti–CD3-PerCP. In parallel, samples were incubated with anti-LILRB1, anti-CD3 and anti-CD56. After washing, erythrocytes were lysed. It is of note that in our first original report the analysis was restricted to the $NKG2C^{bright}$ cell subset as indicated [3], whereas total NKG2C+ lymphocytes were considered in the present study. Protocol 2: PBMC were isolated by centrifugation on Ficoll-Hypaque (Axis Shield PoC AS). Indirect immunofluorescence staining was performed with anti-LILRB1 mAb and a FITC-tagged $F(ab')_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–CD56-APC, CD3-PerCP and anti-CD4 or CD8-PE. Protocol 3: PBMC were isolated and an indirect immunofluorescence staining was performed with anti-LILRB1 unconjugated mAb and washed cells were labeled with FITC-tagged $F(ab')_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–CD8-PE, CCR7-PE-Cy7 and CD45RA-APC. Protocol 4: PBMC were isolated and an indirect immunofluorescence staining was performed with anti-CD27 unconjugated mAb and washed cells were labeled with PE-tagged $F(ab')_2$ rabbit anti–mouse Ig antibody; subsequently, samples were incubated with anti–LILRB1-FITC, CD3- PerCP and CD56-APC, and were analyzed by

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flow cytometry (FACSCalibur; Becton Dickinson). For the sake of precision, flow cytometry data analysis was performed by a single researcher (NR).

Carotid artery ultrasound

B-mode ultrasound imaging of the carotid arterial walls was used to non-invasively assess intima-media thickness according to standardized and validated imaging and image analysis protocols. These protocols have been described in detail elsewhere [4]. In summary, ultrasound communication infrastructure in Girona was set up and sonographers trained and certified by AMC Vascular Imaging (VI), Amsterdam, The Netherlands. An Acuson Aspen with an L7 5-12MHz linear array vascular transducer (Siemens, Erlangen, Germany) was used. A specifically designed REGICOR scan application protocol was developed for the ultrasound equipment to ensure standardization throughout the study. In this application protocol images, clips and demographic information is incorporated into DICOM (Digital Imaging and Communications in Medicine) files as to provide a secure and efficient handling and tracebility record of clinical information. Subjects were scanned in the supine position. Bilaterally, from a single latero-lateral transducer angle, the far walls of the common carotid were imaged by B-mode ultrasound. High resolution stills as well as a dynamic clips of each segment were saved. All images were analysed off-line, centrally in Amsterdam using the eTrack image analysis program. Primary endpoint of the ultrasound study is the per subject average mean common carotid IMT (CIMT).

Statistical analysis

Normality plots were used to assess whether a continuous variable followed a normal distribution or not. Continuous normal distributed variables were summarized as means and standard deviations; continuous non-normal distributed variables were summarized as medians and first and third quartiles; categorical variables were presented as absolute frequencies and proportions. Student's t test was used to compare means for normal distributed variables and Mann-Whitney U test was used to compare medians for continuous non-normal distributed variables. Chi-squared test or exact Fisher test were used as appropriate to compare proportions. Spearman correlation was used to assess the association between continuous variables.

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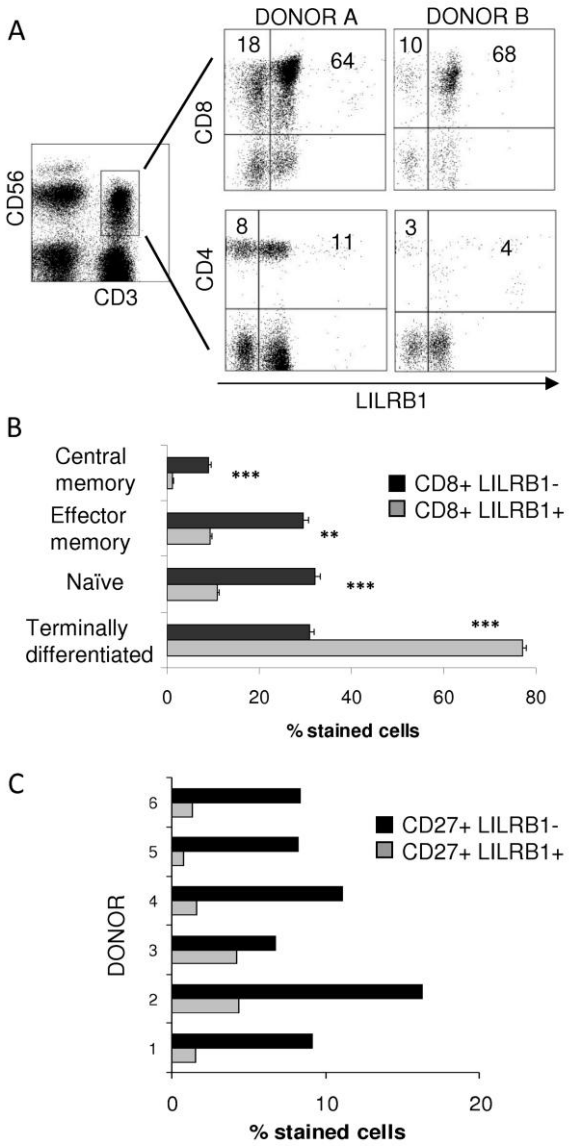
To estimate adjusted means, multivariate linear regression was fitted. A logarithmic transformation was done if the response variable distribution departed from normality.

For age- and sex-matched case-control analysis, an algorithm was applied with the following criteria: every case was matched to two controls of the same sex and with a similar age (± 5 years).

Statistical significance was set up at p-values<0.05. Statistical analysis was done with R software, version 2.10.1.

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Supplemental Figures



Supplemental Figure I. Phenotypic characterization of LILRB1+ T and NK cells. (A) PBMC were stained with anti-LILRB1, anti-CD3, anti-CD56 and either anti-CD4 or CD8 mAbs (protocol 2; see "Immunofluorescence and flow cytometry analysis"). LILRB1 expression was analyzed gating on CD3+ CD56+ cells. The staining pattern observed in two representative donors out of 16 performed is displayed. **(B)** PBMC were also stained with anti-LILRB1, anti-CD8, anti-CD45RA and anti-CCR7 mAbs (protocol 3; see "Immunofluorescence and flow cytometry analysis"). CD45RA and CCR7 expression was analyzed gating on CD8+ LILRB1+ and CD8+ LILRB1- T cells. The proportions (mean ± SEM) of central memory (CD45RA- CCR7+), effector memory (CD45RA- CCR7-), naïve (CD45RA+ CCR7+) and terminally

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differentiated (CD45RA+ CCR7-) CD8+ LILRB1+ and CD8+ LILRB1- T cells in PBMC samples from 16 different donors is shown. ** P<.01, *** P< .001. **(C)** NK cells were also analysed with anti-LILRB1, anti-CD27, anti-CD3 and anti-CD56 (protocol 4; see "Immunofluorescence and flow cytometry analysis"). CD27 expression was analyzed gating on LILRB1+ and LILRB1- NK cells. Data from an analysis representative of six donors analysed is shown.

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Supplemental Tables

Supplemental Table I. Descriptive analysis of NKR expression comparing age and sex-matched HCMV positive and negative individuals

	Subset*	HCMV-negative N=37	HCMV-positive N=148	p-value	Adjusted p-value ‡
	$CD3^+ CD56^-$	72,3 (67,3-77,9)	76,1 (66,1-82,3)	0,223	0.203
	$CD3^+ CD56^+$	1,16 (0,57-1,55)	2,64 (1,35-4,73)	**<0,001**	**<0.001**
	NK	9,88 (6,16-12,9)	6,93 (3,68-12,2)	0,129	0.094
NKG2A *†	$CD3^+$	0,88 (0,34-2,11)	1,16 (0,38-2,80)	0,334	0.407
	$CD3^+ CD56^-$	2,22 (1,05-3,54)	2,07 (1,29-3,55)	0,772	0.618
	$CD3^+ CD56^+$	14,3 (4,32-27,1)	8,42 (3,50-18,9)	0,179	0.178
	NK	33,4 (27,8-41,6)	30,1 (21,7-41,7)	0,099	0.196
NKG2C *†	$CD3^+$	0,91 (0,36-2,50)	1,65 (0,92-3,21)	**0,029**	**0.018**
	$CD3^+ CD56^-$	0,30 (0,09-0,74)	0,49 (0,18-1,12)	**0,032**	**0.023**
	$CD3^+ CD56^+$	1,64 (0,60-4,82)	4,97 (1,53-14,8)	**0,001**	**0.011**
	NK	3,80 (2,44-7,98)	7,69 (4,19-14,5)	**<0,001**	**0.015**
LILRB1 *†	$CD3^+$	2,57 (1,30-4,37)	5,76 (2,82-10,8)	**<0,001**	**<0.001**
	$CD3^+ CD56^-$	1,70 (0,93-3,44)	4,14 (2,27-7,52)	**<0,001**	**<0.001**
	$CD3^+ CD56^+$	10,0 (4,83-25,4)	25,5 (13,2-50,2)	**<0,001**	**<0.001**
	NK	14,9 (9,29-22,7)	17,8 (11,6-34,9)	**0,019**	**0.036**

* Data are expressed as the proportions (%) of cells expressing every receptor within the indicated lymphocyte subsets.

† Data are expressed as median (1^{st}-3^{rd} quartiles). Statistical analysis according to the Mann-Whitney test.

‡ Adjusted by informed of HTA, informed of high glucose and smoking.

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