Expansion of the NKG2C+ Natural Killer–Cell Subset Is Associated With High-Risk Carotid Atherosclerotic Plaques in Seropositive Patients for Human Cytomegalovirus

Jose Enrique Martínez-Rodríguez, Jessica Munné-Collado, Raquel Rasal, Elisa Cuadrado, Luis Roig, Angel Ois, Aura Muntasell, Teresa Baro, Francesc Alameda, Jaume Roquer, Miguel López-Botet

Objective—Human cytomegalovirus (HCMV), a pathogen involved in the development and progression of atherosclerosis, promotes in some individuals a marked reconfiguration of the natural killer (NK)–cell compartment whose hallmark is a persistent expansion of a peripheral blood NK-cell subset expressing the CD94/NKG2C NK receptor. We aimed to evaluate whether the HCMV-associated NK-cell compartment reconfiguration is related to carotid atherosclerotic plaque (CAP) instability.

Approach and Results—NK receptor expression (ie, LILRB1, NKG2A, NKG2C, and killer immunoglobulin-like receptors) by peripheral NK and T cells was evaluated in 40 patients with HCMV+ with CAP, including nonatherosclerotic strokes (n=15) and healthy subjects (n=11) as controls. High-risk CAP (n=16), defined as carotid stenosis >50% with ipsilateral neurological symptomatology in the previous 180 days, compared with non–high-risk CAP had higher %NKG2C+ NK cells (29.5±22.4% versus 16.3±13.2%; P=0.026; odds ratio, 1.053; 95% confidence interval, 1.002–1.106; P=0.042), with a corresponding reduction in the NKG2A+ NK subset (31.7±17.8% versus 41.8±15.8%; P=0.072). The proportions of NKG2C+ NK cells in high-risk CAP were inversely correlated with the CD4+/CD8+ ratio (R_spearman=−0.629; P=0.009) and directly with high-sensitivity C-reactive protein levels (R_spearman=0.591; P=0.012), consistent with higher subclinical systemic inflammation. The intraplaque inflammatory infiltrate, evaluated in 27 CAP obtained after endarterectomy, showed a higher presence of subintimal CD3+ lymphocytes in those patients with HCMV-induced changes in the peripheral NK- and T-cell compartments.

Conclusions—The expansion of NKG2C+ NK cells in patients with CAP seems to be associated with an increased risk of plaque destabilization in some patients with chronic HCMV infection. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: carotid artery diseases ■ human cytomegalovirus ■ killer cells, natural ■ NKG2C receptor

Atherosclerotic plaques in the carotid arteries are a strong predictor of stroke and cardiovascular disease. Yet, some carotid atherosclerotic plaques (CAPs) are stable and unlikely to produce symptoms, whereas in other patients they may lead to increased incidence of thrombosis and rapid stenosis, thus being considered of high risk. Previous studies and current guidelines suggest that endarterectomy, that is, surgical removal of the CAP, should be indicated based on the degree of stenosis and the presence of previous symptoms. Symptomatic plaques are usually present as intima rupture, a thinner fibrous cap, and a greater inflammatory infiltrate of macrophages and lymphocytes. However, pathophysiological processes leading to plaque instability are not well understood. Defining biomarkers associated with high-risk CAP would become especially useful to establish therapeutic procedures before the development of clinical events, identifying candidates for early endarterectomy.

Infections are considered a potential trigger of immune mechanisms leading to initiation and acceleration of atherosclerosis and the development of cerebrovascular complications. No single microbial pathogen has been unequivocally identified as a direct cause of atherosclerosis, but evidence has been obtained supporting that the global impact of past and chronic infections may contribute to the inflammatory process on the atherosclerotic plaque and increase the risk of vascular complications. Such infectious burden has been previously estimated based on the titers of pathogen-specific
antibodies, which only partially reflect the complexity of the host–pathogen relationship.

In this context, herpesviruses may have a substantial effect in the progression of atherosclerosis and the cardiovascular risk. Among them, human cytomegalovirus (HCMV) is thought to be involved in the development of atherosclerosis based on clinico-epidemiological and experimental studies and has been proposed to contribute to the progression of the carotid plaque thickness and the degree of stenosis. In healthy individuals, HCMV remains latent, establishing a persistent infection and eventually undergoing subclinical reactivations. A remarkable fraction of the CD8+ T-cell compartment may be directed against HCMV, a phenomenon that has been associated with immunosenescence, leading in some aged healthy individuals to a reduction of the CD4+/CD8+ T-cell ratio. In addition, HCMV has been shown to promote a persistent expansion of a peripheral blood NK-cell subset expressing high levels of the CD94/NKG2C+ lectin-like receptor, detectable to a variable degree in healthy subjects. We hypothesize that the contribution of HCMV infection to atherosclerosis may depend on features of the host–pathogen relationship not reflected by the simple serological status. In the present study, the relationship of the HCMV-associated NK-cell compartment reconfiguration with CAP instability was assessed. We found that a subset of high-risk patients with CAP displayed increased NKG2C+ NK cells and related changes in the peripheral CD8+ T-cell compartment that were associated with biochemical and pathological findings suggestive of subclinical inflammation.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Clinical Characteristics of Patients and Controls
As detailed in the Methods in the online-only Data Supplement, after exclusion of patients with HCMV− with CAP (2 high-risk CAP and 1 non–high-risk CAP) and NKG2C−/− subjects (n=1), we evaluated 39 patients with HCMV+ with CAP that were classified as high risk (n=16) and non–high risk (n=23).

The Table summarizes the main clinical characteristics of patients with HCMV+ and controls. Ischemic heart disease and peripheral vascular disease had a similar prevalence in high-risk and non–high-risk CAP, suggesting a comparable systemic extension of the atherosclerotic process (Table I in the online-only Data Supplement). No differences were

Table. Clinical Characteristics of Patients With CAP, Nonatherosclerotic Stroke, and Controls

<table>
<thead>
<tr>
<th></th>
<th>High-Risk CAP (n=16)</th>
<th>Non–High-Risk CAP (n=23)</th>
<th>P Value</th>
<th>Nonatherosclerotic Stroke (n=15)</th>
<th>P Value</th>
<th>Controls (n=11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>71.4 (9.7)</td>
<td>69.8 (9.3)</td>
<td>0.610</td>
<td>72.6 (12.2)</td>
<td>n.s.</td>
<td>67.7 (6.7)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Women</td>
<td>5</td>
<td>6</td>
<td>0.500</td>
<td>5</td>
<td>n.s.</td>
<td>7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Body mass index</td>
<td>28.53 (6.35)</td>
<td>28.93 (2.98)</td>
<td>0.821</td>
<td>26.43 (4.55)</td>
<td>n.s.</td>
<td>25.35 (3.07)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Smoker</td>
<td>10 (67%)</td>
<td>16 (73%)</td>
<td>0.484</td>
<td>5 (33%)</td>
<td>0.072*</td>
<td>4 (36%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hypertension</td>
<td>12 (75%)</td>
<td>19 (83%)</td>
<td>0.425</td>
<td>11 (73%)</td>
<td>n.s.</td>
<td>5 (45%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>9 (56%)</td>
<td>8 (35%)</td>
<td>0.158</td>
<td>4 (27%)</td>
<td>0.096*</td>
<td>2 (18%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>10 (62%)</td>
<td>18 (78%)</td>
<td>0.237</td>
<td>7 (47%)</td>
<td>n.s.</td>
<td>6 (54%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>2 (12%)</td>
<td>5 (22%)</td>
<td>0.383</td>
<td>3 (20%)</td>
<td>n.s.</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>4 (25%)</td>
<td>9 (39%)</td>
<td>0.285</td>
<td>0</td>
<td>0.058*</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Days from stroke</td>
<td>33.9 (42.4)</td>
<td>…</td>
<td>…</td>
<td>2 (1.4)</td>
<td>0.007*</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>NIHSS at stroke onset</td>
<td>5.13 (4.6)</td>
<td>…</td>
<td>…</td>
<td>8.13 (6.3)</td>
<td>n.s.</td>
<td>…</td>
<td>…</td>
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<tr>
<td>Plaque stenosis</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>50–70%</td>
<td>3</td>
<td>3</td>
<td>0.478</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>&gt;70%</td>
<td>13</td>
<td>20</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>hs-CRP levels, mg/dL</td>
<td>0.27 (0.39)</td>
<td>0.17 (0.26)</td>
<td>0.369</td>
<td>0.61 (1.21)</td>
<td>n.s.</td>
<td>0.03 (0.03)</td>
<td>0.003‡‡</td>
</tr>
<tr>
<td>HCMV IgG titer, aU/mL</td>
<td>55.5 (21.5)</td>
<td>55.3 (37.6)</td>
<td>0.982</td>
<td>51.2 (24.2)</td>
<td>n.s.</td>
<td>59.5 (43)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Endarterectomy</td>
<td>11 (69%)</td>
<td>17 (74%)</td>
<td>0.500</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
</tbody>
</table>

Statistical analysis is according to Student t test; values are expressed as mean (SD). CAP indicates carotid atherosclerotic plaque; HCMV, human cytomegalovirus; hs-CRP, high-sensitivity C-reactive protein; NIHSS, National Institute of Health Stroke Scale; and n.s., nonsignificant.

*P value comparing high-risk CAP with nonatherosclerotic stroke.
†P value comparing high-risk CAP with controls.
‡P value comparing non–high-risk CAP with controls.
§P value comparing nonatherosclerotic stroke with controls.
||Significant P value (P<0.05).
Increased NKG2C Expression by NK Cells in Patients With High-Risk CAP

NK receptor (LILRB1, NKG2A/C, and killer immunoglobulin-like receptors) expression by NK and T cells in patients and controls is summarized in Table I in the online-only Data Supplement. As expected, NKG2C expression by NK cells in patients with HCMV− with CAP was low (%NKG2C+ NK cells, 4±1.7). Once HCMV− subjects were excluded, as compared with the other groups, NK cells from patients with high-risk CAP displayed significantly higher %NKG2C+ and lower %NKG2A+ NK cells (Figure 1A and 1B). The proportions of NKG2C+ NK cells displayed a dichotomous distribution in high-risk patients with CAP that was not observed in other groups (Figure 1). Establishing a cut-off value in the higher tertile of %NKG2C+ NK cells allowed differentiation of high-risk CAP from non–high-risk CAP with a sensitivity ≤56% and specificity of 73% (odds ratio, 1.053; 95% confidence interval, 1.002–1.106; P=0.042).

No differences in the proportions of CD56dim, CD56bright NK cells, or CD56− NK-cell subsets were found (data not shown). In contrast to NK cells, the expression of NK receptor by CD8+, CD4+, and CD56+ T lymphocytes was not significantly different according to CAP stability or clinical characteristics of patients, except for LILRB1 expression by CD4+ T lymphocytes (Table I in the online-only Data Supplement). Altogether, these results suggest that the magnitude of the NK-cell compartment reconfiguration by HCMV is directly related to the risk of plaque instability in patients with CAP.

Relationship of High-Sensitivity C-Reactive Protein With NKG2C Expression in CAP

As previously described,15 high-sensitivity C-reactive protein (hs-CRP) levels were elevated in patients with CAP and stroke as compared with controls (Table), without significant differences between patients with high-risk or non–high-risk CAP. Yet, hs-CRP levels in high-risk CAP seemed to be directly related to the %NKG2C+ NK cells (RPearson=0.591; P=0.012) and inversely related to the CD4+/CD8+ ratio (RPearson=−0.59; P=0.017). Such associations were not found in non–high-risk CAP, nonatherosclerotic strokes, or controls. Patients with symptomatic CAP and nonatherosclerotic stroke had no different hs-CRP levels or NKG2C expression according to the time after stroke onset (data not shown), thus ruling out any...
possible association of higher NKG2C expression with the acute phase of stroke. Moreover, %NKG2C+ NK cells were not related to clinical severity evaluated by the National Institute of Health Stroke Scale at stroke onset in high-risk CAP (R_spearman=−0.166; P=0.615). Altogether, these results suggest that patients with high-risk CAP display a subclinical inflammation, as indicated by the higher levels of hs-CRP, that is associated with HCMV-related changes in NK and T cells.

Magnitude of the T-Cell Infiltrate in the CAP Subintimal Region Is Associated With Changes in Peripheral NK and CD8+ T Cells

According to the histopathologic analysis of 27 CAP obtained by endarterectomy (11 high risk and 16 non–high risk), lesions were classified as type VII (calcification predominance, 7 cases) and type VIII (fibrous tissue changes predominance, 20 cases). Arterial intima ulceration was identified in 17 cases (63%), rupture of elastic fibers in 26 cases (96%), calcification in 23 cases (85%), and lipid deposits in 14 cases (52%). None of the previous immunologic findings were related to any macroscopic variables of the CAP.

The cellular inflammatory infiltrate at the sinus of the arterial wall has been proposed to contribute to the initiation, progression, and rupture of the plaque, leading to vascular complications. Thus, a quantitative analysis of the CD3+ infiltrate in the subintima region was evaluated in relation with CAP stability. The magnitude of the T-cell infiltrate showed a wide variability (Figure 3A and 3B), and no differences between high-risk and non–high-risk CAP (16.9±8.4 cells/field versus 17±8.7 cells/field, respectively) were noticed. However, the T-cell infiltrate inversely correlated with the proportions of peripheral NK cells and seemed to be directly related to the absolute and relative numbers of peripheral CD8+ T lymphocytes (Figure 3C), with a decreased CD4+/CD8+ ratio in those CAP with a greater CD3+ infiltrate (Figure 3E). No relation with peripheral CD4+ T lymphocytes was observed (Figure 3C). Analyzing the NK-cell compartment, the proportions of NKG2A+ NK cells were inversely related to the CD3+ infiltrate (R_Pearson=−0.390; P=0.044; Figure 3D). No significant relationship was detected for NKG2C+ NK cells and the CD3+ infiltrate in the whole group (Figure 3D), but patients with high-risk CAP with a %NKG2C+ NK cells in the third tertile showed a trend for higher T-cell infiltrate compared with those CAP with NKG2C in the lower tertiles (21.1±8.8 versus 14.9±7.6; P=0.076). Altogether, these results suggest a relation between the intraplaque T lymphocyte infiltrates with changes in the peripheral NK- and CD8+ T-cell compartments.

The infiltrate of CD68+ macrophages (mean, 13.2±4.8 cells/field) was not associated with CAP stability or changes in NK- and T-cell populations. The minimal presence of CD56+ cells within the CAP (0.5±0.39 cells/field; Figure 3F) was in accordance with previous reports detecting a scarce number of NK cells in the core of atherosclerotic plaques, thus suggesting that NK cells are not directly involved in situ plaque instability.

Discussion

The complex pathophysiology of atherosclerosis and the multiple pathogenic factors involved have hampered the development of biomarkers useful for detection of vulnerable patients with unstable plaques at higher risk of vascular complications. We report that a subset of patients with HCMV+ with CAP considered of high risk, based on the stenosis degree and the presence of neurological symptoms in the previous months, displayed high proportions of peripheral NKG2C+ NK cells. Together with additional immunologic and pathological features observed in these patients, the data suggest that an increased NKG2C expression associated with HCMV infection might be of potential value for predicting high-risk CAP. Despite that higher proportions of NKG2C+ NK cells were predominantly found in a subset of high-risk CAP, this phenotypic feature was also observed in some patients with nonatherosclerotic stroke and in healthy controls, in agreement with previous reports. These findings should be interpreted in the framework of the current view on the contribution of infectious agents to the pathogenesis of atherosclerosis.
thickness). On that basis, several reports have proposed that the infectious burden contributes as an independent risk factor to vascular lesions, resulting from a cumulative proinflammatory impact on the arterial wall of different chronic/recurrent infections. However, studies based on the clinical outcome of atherosclerosis may not discriminate to what extent the infectious insult contributes to the development of vascular lesions or promotes the instability of plaques, which may have been formed because of other risk factors as well. In this context, HCMV is a usual suspect, and several hypothetical mechanisms have been proposed to explain its contribution in the pathogenesis of atherosclerosis, for example, induction of endothelial damage, homing of latently infected monocytes, direct infection of different cell types in the vessel wall, and triggering of antiviral immune responses.

Presumably, the impact of HCMV on atherosclerosis development may be particularly relevant when the latent infection is inefficiently controlled, as observed in immunosuppressed patients. In this regard, NK cells are involved in the immune defense against HCMV together with T lymphocytes. NK-cell functions are controlled by a balance between inhibitory and activating signals triggered by different surface NK-cell receptors. The inhibitory CD94/NKG2A NK receptor, specific for HLA-E, contributes to monitor HLA-I expression together with killer immunoglobulin-like receptors. By contrast, the biological function of the activating CD94/NKG2C NK receptor, which also recognizes HLA-E, remains unclear. Remarkably, a persistent expansion of NKG2C+ NK cells has been reported in healthy HCMV+ individuals, in immunosuppressed kidney transplant, and

Figure 3. Relationship between the intraplaque inflammatory infiltrate and the distribution of peripheral natural killer (NK)-cell and T-cell subsets. Representative cases of carotid atherosclerotic plaque (CAP) with a high (A) and low inflammatory infiltrate of CD3+ in the subintima area (B). The CD3+ infiltrate was inversely correlated with peripheral NK cells and directly with CD8+ T cells, with no relationship found for CD4+ T cells (C). Correlations of the CD3+ infiltrate with NKG2A and NKG2C expression by peripheral NK cells (D) and the CD4+/CD8+ ratio (E). NK cells (CD56+) were scarce in CAP and predominantly found in the periphery of the plaque (F). The infiltrate of CD3+ is expressed as cells per high-power field (*P<0.05; Pearson correlation). AN indicates absolute numbers.
in hematopoietic cell transplantation after HCMV reactivation. Increased numbers of NKG2C+ NK cells detected in the course of other infections (eg, HIV, hepatitis B virus/ hepatitis B virus, Hantavirus, and Chikungunya virus) have been systematically associated with HCMV coinfection. In our study, we cannot ascertain whether the immunophenotypic changes associated with high-risk CAP indeed precede the development of the acute episode. However, our results do not support changes in NKG2C expression related to the acute phase of stroke. Furthermore, recent studies have shown that the NKG2C+ NK-cell expansion may occur early in childhood and the phenotype seems to remain stable over time. Furthermore, recent studies have shown that changes in NKG2C expression related to the acute phase of stroke. However, our results do not support changes in NKG2C expression related to the acute phase of stroke. Furthermore, recent studies have shown that the NKG2C+ NK-cell expansion may occur early in childhood and the phenotype seems to remain stable over time. However, our results do not support changes in NKG2C expression related to the acute phase of stroke. Furthermore, recent studies have shown that the NKG2C+ NK-cell expansion may occur early in childhood and the phenotype seems to remain stable over time.

In this context, the increased %NKG2C+ NK cells, and the corresponding reduction of the NKG2A+ subset, detected in the group of patients with high-risk CAP, are consistent with an HCMV-mediated reconfiguration of the NK-cell compartment, which, moreover, seemed to be associated with parameters of systemic and intraplaque subclinical inflammation. In this regard, higher levels of hs-CRP, a marker related to systemic inflammation, cardiovascular risk, and the infectious burden, were detected in patients with high-risk CAP displaying increased NKG2C+ NK cells. Moreover, CAP lesions with a greater intraplaque infiltration of T cells (ie, with an increased risk of complications) corresponded to cases with lower %NKG2A+ NK cells and higher peripheral CD8+ T cells. Patients with increased proportions of NKG2C+ NK cells also had greater T-cell infiltrates although this finding did not reach statistical significance ($P=0.076$), possibly because of the limited sample size. In our study, peripheral CD8+ T lymphocytes were not related to the intraplaque inflammatory infiltrate, a finding that has to be interpreted assuming a concomitant statin therapy in most of the patients (77%), which may have modified the peripheral numbers of these lymphocytes, as previously reported.

Of note, a previous study did not reveal any significant association of NKG2C expression with either acute myocardial infarction or carotid intima-media thickness in control subjects. These results, which did not rule out a contribution of HCMV to the pathogenesis of atherosclerosis, are not in conflict with the present study in which the HCMV-induced expansion of NKG2C+ cells was associated with CAP instability, a process different from plaque development assessed by measuring carotid intima-media thickness. However, the lack of relation between NKG2C+ NK cells and acute myocardial infarction may reflect differences between the evolution of carotid and coronary atherosclerosis related to factors such as diameter, hydrostatic pressure, and flow turbulences, which may underlie differences in clinical expressivity (eg, age at first clinical presentation and vascular complications). A direct role of NKG2C+ NK cells in plaque instability can be ruled out as NK cells were scarce in infiltrates, in agreement with previous reports.

High-risk CAP displayed higher numbers of peripheral CD8+ T cells, related to the NKG2C expression by NK cells and the presence of intraplaque CD3+, suggesting that T lymphocytes may be involved in plaque instability. Assessing the antigen specificity of T cells in CAP from patients exhibiting high levels of peripheral NKG2C+ NK cells would be required to formally establish a link with HCMV infection.

In conclusion, our study reveals HCMV infection–related changes in the NK-cell compartment in patients with high-risk CAP that seemed to be related to increased peripheral CD8+ T cells and to systemic and intraplaque subclinical inflammation. The expansion of NKG2C+ NK cells in patients with CAP may be associated with an increased risk of plaque destabilization in some patients with chronic HCMV infection. Further studies are warranted to establish whether these findings may be useful for the identification of patients with vulnerable atherosclerotic plaques.

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**Disclosures**

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