Circulating CD40+ and CD86+ B Cell Subsets Demonstrate Opposing Associations With Risk of Stroke

Polyxeni T. Mantani, Irena Ljungcrantz, Linda Andersson, Ragnar Alm, Bo Hedblad, Harry Björkbacka, Jan Nilsson, Gunilla Nordin Fredrikson

Objective—Accumulating evidence shows that immune cells play an important role in atherosclerosis. Most attention has focused on the role of different T cell subsets, whereas the possible involvement of B cells has been less studied. In this study, we assessed the association of 2 different B cell subsets, CD19+CD40+ and CD19+CD86+ B cells, with risk for development of acute cardiovascular events.

Approach and Results—The prospective study included 700 subjects randomly selected from the cardiovascular cohort of the Malmö Diet and Cancer study. Mononuclear leukocytes, stored at −140°C at the baseline investigation in 1991–1994, were thawed and B cell subsets analyzed by flow cytometry. Cytokine release from CD3/CD28-stimulated mononuclear leukocytes was measured with multiplex ELISA. Baseline carotid intima-media thickness and stenosis were assessed by ultrasonography, and clinical events were monitored through validated national registers during a median/mean follow-up time of 15 years. The subjects in the highest tertile of CD19+CD40+ B cells had a significantly lower risk of incident stroke after adjustment for other risk factors. In contrast, CD19+CD86+ B cells were associated with higher risk for development of a stroke event and increased release of proinflammatory cytokines from mononuclear leukocytes.

Conclusions—These observations provide evidence for an involvement of B cells in the incidence of stroke and suggest that both pathogenic and protective B cell subsets exist. (Arterioscler Thromb Vasc Biol. 2014;34:211-218.)

Key Words: B-lymphocyte subsets  ■ carotid artery diseases  ■ prospective studies  ■ stroke
B cell deficiency in murine models of autoimmune disease has led to worsened outcomes, suggesting that B cells also could have regulatory roles. In this context, studies have explored the role of different costimulatory molecules. It has been shown that stimulation of the costimulatory molecule CD40 brings about development of B cells with suppressive properties, and that absence of CD40 makes B cells unable to regulate inflammatory immune responses. There is evidence that the effect of the CD40 engagement on B cells depends on the maturation of the B cell as well as on the duration and strength of the signals between T and B cells, and thereby result in either regulatory or antibody producing B cells. Furthermore, regulatory interleukin (IL)-10 producing B cells activated in a T cell CD40-dependent manner have been demonstrated to suppress Th1 cell responses and prevent the induction of autoimmune disease in several mouse models. Regulation of the proliferation of human T cells has been shown to require only cell–cell contact involving CD40 engagement, whereas Th1 cell differentiation is dependent on CD80/CD86 interactions and IL-10 production. Expression of CD80 and CD86 by B cells was found to be required for the activation of autoreactive CD4+ T cells in experimental arthritis. However, signaling through CD80 and CD86 has in other studies been proposed to take part in the suppressive effect of B cells, making the picture of these costimulatory molecules more complicated. Studies of regulatory B cells in humans have demonstrated a CD19+CD40hiCD86hi B cell subset producing IL-10 in high density lipoprotein (LDL)–DR on both CD19+CD40+ and CD19+CD86+ B cells after adjustment for the fraction of CD3+ T cells, whereas release of proinflammatory cytokines with the exception of interferon-γ associated with the fraction of CD19+CD86low B cells (Table 2). Because the mononuclear cells were stimulated with anti-CD3/CD28 beads, also associations between the cytokine release and the fraction of CD3+ T cells were calculated. The T cells were found to correlate inversely with the release of tumor necrosis factor (TNF)-α, IL-10, and IL-5 (Table 2). Additionally, a high median fluorescence intensity of inducible costimulator ligand (ICOSL) on CD19+CD86+ B cells correlated with lower levels of released cytokines, whereas a high median fluorescence intensity of human leukocyte antigen (HLA)-DR on both CD19+CD40+ and CD19+CD86+ B cells corresponded to a proinflammatory cytokine profile (Table 3).

B Cell Subsets and CVD

In the present study, viable CD19+ B cells and the viable CD19+CD40+ and CD19+CD86+ B cell subsets were investigated (Figure 1). No correlation between B cell subsets and common and bulb carotid intima-media thickness was detected, but high numbers of CD19+CD86+ B cells correlated with more stenosis (r=0.110; P=0.012) after adjustment for common risk factors (age, sex, LDL/high-density lipoprotein ratio, blood glucose, and systolic blood pressure). In a logistic regression model, no association was found between B cell subsets and presence of carotid plaques. The B cell subsets were further analyzed in relation to coronary and stroke events. The analysis revealed a significantly smaller fraction of CD19+CD40+ B cells in individuals with a later incidence of stroke compared with controls, and the stroke cases group also displayed elevated percentages and numbers of CD19+CD86+ B cells compared with controls (Table 1). No differences
were detected in percentages or numbers of the B cell subsets between coronary cases and controls (Table 1). In the next step, the percentages and numbers of the B cell subsets were divided into tertiles. In analysis of the tertiles of both the fractions and the numbers of the B cell subsets, there was a significant connection between more CD40+ B cells and less CD86+ B cells and vice versa ($P$ for linear trend <0.001 for both the fractions and the numbers), suggesting that they are compensating for each other. To determine whether there were time-dependent associations between B cell subsets and risk of stroke events, the tertiles of the B cell subsets were plotted into Kaplan–Meier curves. The survival curves revealed a significant positive linear trend over tertiles of the fraction of CD19+CD40+ B cells (log-rank [Mantel Cox] test; $P$ for linear trend <0.01; Figure 2A) and the tertiles of the fraction as well as numbers of CD19+CD86+ B cells (log-rank [Mantel Cox] test; $P$ for linear trend <0.05; Figure 2C; $P$ for linear trend <0.05; Figure 2D, respectively). No significant linear trend was found over tertiles of the numbers of CD19+CD40+ B cells (log-rank [Mantel Cox] test; $P$ for linear trend=0.25; Figure 2B). After exclusion of the 11 hemorrhagic stroke individuals, the significant positive linear trend over tertiles remained for the fraction of CD19+CD40+ B cells (log-rank [Mantel Cox] test; $P$ for linear trend <0.01), whereas it disappeared for tertiles of numbers as well as for the fraction of CD19+CD86+ B cells (log-rank [Mantel Cox] test; $P$ for linear trend=0.14; and $P$ for linear trend=0.08, respectively). The tertiles of the fraction of CD19+CD40+ B cells were thereafter entered into a Cox proportional hazard regression model. A significant association was identified between a high fraction of this B cell subset and lower risk of incidence of stroke after adjustment for risk factors as age, sex, LDL/high-density lipoprotein.

### Table 1. Baseline Clinical Characteristic of the Study Cohort

<table>
<thead>
<tr>
<th></th>
<th>All Noncases (n=549)</th>
<th>Coronary Cases (n=84)†</th>
<th>Stroke Cases (n=66)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at screening, y</td>
<td>65.6±1.1</td>
<td>65.7±1.2</td>
<td>65.5±1.3</td>
</tr>
<tr>
<td>Sex (% men)</td>
<td>38.3</td>
<td>53.6*</td>
<td>54.9*</td>
</tr>
<tr>
<td>BMI</td>
<td>26.3±4.0</td>
<td>26.6±4.1</td>
<td>26.5±3.6</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>16.0</td>
<td>25.3</td>
<td>26.9</td>
</tr>
<tr>
<td>Diabetes mellitus, %‡</td>
<td>11.1</td>
<td>25.0***</td>
<td>16.9</td>
</tr>
<tr>
<td>Hypertension, %§</td>
<td>79.4</td>
<td>86.9</td>
<td>88.7</td>
</tr>
<tr>
<td>Medication, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antidiabetic</td>
<td>2.3</td>
<td>4.7</td>
<td>9.9**</td>
</tr>
<tr>
<td>Lipid lowering</td>
<td>2.9</td>
<td>4.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Blood pressure lowering</td>
<td>20.4</td>
<td>33.3*</td>
<td>38.0**</td>
</tr>
<tr>
<td>Laboratory parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting venous blood glucose</td>
<td>5.3±1.3</td>
<td>5.7±1.8</td>
<td>5.7±2.0</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.5±0.8</td>
<td>1.5±0.9</td>
<td>1.6±0.7</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.4±0.4</td>
<td>1.3±0.4*</td>
<td>1.3±0.4*</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>4.3±1.0</td>
<td>4.4±1.0</td>
<td>4.1±1.2</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>3.4±1.1</td>
<td>3.7±1.4</td>
<td>3.6±1.5</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>150±20</td>
<td>156±21*</td>
<td>153±18</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>88±9</td>
<td>89±8</td>
<td>90±9*</td>
</tr>
<tr>
<td>high-sensitive CRP, mg/L</td>
<td>2.9±5.0</td>
<td>4.5±8.1</td>
<td>4.2±7.2</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.0±0.8</td>
<td>5.2±0.9</td>
<td>5.3±1.1*</td>
</tr>
<tr>
<td>White blood cells (×10⁶ cells/mL)</td>
<td>6.1±1.6</td>
<td>6.4±1.7</td>
<td>6.3±1.6</td>
</tr>
<tr>
<td>Lymphocytes (×10⁶ cells/mL)</td>
<td>1.83±0.57</td>
<td>1.77±0.58</td>
<td>1.81±0.58</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD19+ B cells of lymphocytes</td>
<td>13.5±5.8</td>
<td>13.5±5.0</td>
<td>13.6±5.1</td>
</tr>
<tr>
<td>% CD40+ of CD19+ B cells</td>
<td>61.8±15.7</td>
<td>62.9±16.9</td>
<td>55.6±18.3**</td>
</tr>
<tr>
<td>% CD86+ of CD19+ B cells</td>
<td>21.9±11.4</td>
<td>23.3±14.3</td>
<td>25.2±12.1*</td>
</tr>
<tr>
<td>CD19+ B cells, cells/µL</td>
<td>251.7±212.0</td>
<td>233.7±111.6</td>
<td>245.2±116.6</td>
</tr>
<tr>
<td>CD19+CD40+ B cells, cells/µL</td>
<td>156.0±144.6</td>
<td>149.4±84.9</td>
<td>140.1±88.6</td>
</tr>
<tr>
<td>CD19+CD86+ B cells, cells/µL</td>
<td>51.6±34.5</td>
<td>51.7±33.4</td>
<td>59.1±36.2*</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; BP, blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

*P<0.05, **P<0.01, and ***P<0.001 for coronary cases (n=84) vs all noncoronary cases (n=615) and for stroke cases (n=66) vs all nonstroke cases (n=633).

†Mann–Whitney test or χ² test for categorical data.

‡History of diabetes mellitus, medication, or fasting glucose ≥6.1 mmol/L.

§Blood pressure ≥140/90 mm Hg or treatment.
lipoprotein ratio, body mass index, systolic blood pressure, and hemoglobin A1c (hazard ratio [95% confidence interval], 0.53 [0.28, 0.97]; \( P = 0.039 \); Table 4). Furthermore, a significant association between high numbers of CD19+CD86+ B cells and increased risk of stroke was found after adjustment of the model with the same risk factors (hazard ratio [95% confidence interval], 2.4 [1.2, 4.8]; \( P = 0.011 \); Table 4). On the contrary, a high fraction of the CD19+CD86+ B cell subset revealed a significant association to increased risk of incident stroke (hazard ratio [95% confidence interval], 1.4 [1.1, 1.9]; \( P = 0.033 \)), which disappeared when the association was adjusted for age, sex, LDL/high-density lipoprotein ratio, body mass index, systolic blood pressure, and hemoglobin A1c (\( P = 0.081 \); Table 4). After adjustment for prevalent stroke in the Cox proportional hazard regression model, the associations were still significant for both the B cell subsets tested (data not shown). In \( \chi^2 \) analysis of men and women separated, the percentage of CD19+CD40+ and CD19+CD86+ B cell tertiles demonstrated a pattern indicating that most males had high levels of CD19+CD86+ B cells and low levels of CD19+CD40+ B cells, whereas most females showed the opposite (\( P \) for linear trend=0.001 for both CD19+CD40+ and CD19+CD86+ B cells). Taken together, this may indicate that males are at a higher risk of incidence of stroke according to the pattern of the B cell subsets.

The normalized median fluorescence intensity of the surface molecules HLA-DR and ICOSL was not significantly different between cases and controls on any of the B cell subsets (data not shown). However, high surface expression of HLA-DR correlated with less common carotid intima-media thickness (\( r = -0.127 \); \( P = 0.001 \) for CD19+CD40+ B cells and \( r = -0.097 \); \( P = 0.019 \) for CD19+CD86+ B cells).

**Discussion**

B cells are essential for humoral immunity, but their role in regulating T cell responses remains to be fully characterized.
Both human and mouse studies show that the clinical efficacy of B cell depletion therapy may not correlate with changes in levels of circulating autoantibodies, suggesting that B cells may contribute to autoimmunity independently of auto-antibody production.\textsuperscript{32,33} Interestingly, a recent study demonstrated that different B cell subsets may have opposing impact on the development of acute cardiovascular events in a prospective study cohort, involving 700 randomly selected subjects from the cardiovascular arm of MDCS with a median/mean follow-up time of 15 years. The results demonstrated an association between high levels of CD19\textsuperscript{+}CD40\textsuperscript{+} B cells and a decreased risk for incidence of stroke. On the contrary, high numbers of CD19\textsuperscript{+}CD86\textsuperscript{+} B cells were associated with an increased risk of stroke. This suggests that different B cell subsets may have opposing impact on the risk of incident stroke.

Flow cytometric immunophenotyping has been used to study peripheral B cell maturation and differentiation in humans. A suppressive CD19\textsuperscript{hi}IgD\textsuperscript{lo}CD38\textsuperscript{lo}CD24\textsuperscript{hi}CD5\textsuperscript{hi} B cell subset has been identified in humans, and it seemed to have an indirect function by inducing Foxp3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+} T regulatory cells.\textsuperscript{29} Interestingly, the results indicated that B cells activated by T cells through CD40 induced inhibition of T cell proliferation and differentiation to Th1-type cells, whereas the Th1 cell differentiation was dependent on CD80 and CD86 interactions. In experimental studies, a phenotypically distinct CD14\textsuperscript{+}CD5\textsuperscript{+} B cell subset was found to be potent regulatory B cells by controlling T cell dependent inflammatory responses through IL-10 secretion.\textsuperscript{43} Regulatory IL-10–producing B cells suppressing Th1 cell responses have also been demonstrated to prevent induction of autoimmune disease in mouse models.\textsuperscript{2,33} Interestingly, a recent study demonstrated that IL-10–secreting B cells are the major regulatory cell type in experimental stroke.\textsuperscript{44} In humans, the B cell subset CD19\textsuperscript{+}CD24\textsuperscript{hi}CD38\textsuperscript{hi} after CD40 stimulation demonstrated...
regulatory capacity and suppressed the differentiation of Th1 cells, which was also shown to be dependent on the presence of IL-10. In the present study, we were interested in analyzing CD19+ B cells expressing the costimulatory markers CD40 and CD86 because no prospective clinical study has presented involvement of B cells subsets in atherosclerosis development. One of our findings demonstrated that individuals later suffering an incident stroke had lower levels of CD19+CD40+ B cells compared with controls. This is in line with previous findings suggesting that stimulation of CD40 induces development of B cells with suppressive properties, and that absence of CD40 makes B cells unable to regulate inflammatory immune responses. In contrast, higher numbers of the CD19+CD86+ B cell subset were associated with increased risk of incident stroke. The risk of incident stroke was doubled in the presence of high numbers of this B cell subset, and the risk seemed to be higher in men. It is known that B cells acting as antigen-presenting cells upregulate the costimulatory mediator CD86 to interact with T cells that in its turn make the B cells important IgG antibody producers. Thus, antibodies may also contribute to the stroke pathophysiology. In accordance, a previous study demonstrated that high levels of IgG against a defined oxidized LDL epitope reflected structure and disease activity of carotid plaques. Interestingly, it may also reflect previous findings suggesting that Th1 cell differentiation is dependent on CD86 interactions and the evidence from experimental animal studies indicating that Th1 immune responses play an important role in driving atherosclerosis. In accordance, it has been demonstrated that naïve and memory B cells from patients with rheumatoid arthritis express higher percentage of CD86 than healthy controls. In coming studies, it will be of interest to analyze whether any associations exist between CD19+CD40+ or CD19+CD86+ B cell subsets and the previously identified suppressive B cell subsets expressing CD19+CD27+CD123highCD45+CD38highCD10+CD38high or CD19+CD24highCD38high. Interestingly, it has been shown that B cells explore a defective regulation capacity in patients with systemic lupus erythematosus that might depend on ongoing inflammation, a phenomenon also present in patients with CVD. One study observed that B cells from systemic lupus erythematosus patients were unable to regulate T cell proliferation, whereas another demonstrated that CD19+CD24highCD38high B cells isolated from patients with systemic lupus erythematosus did not suppress CD4+ T cell cytokine production, and these B cells were also refractory to further CD40 engagement.

Inflammation and immune mechanisms have been found to influence the pathogenesis of atherosclerosis and coronary artery disease as well as the risk and causation of stroke. The discrepancies found in the present study that only stroke and not coronary artery disease was associated with the B cell subsets may reflect the heterogeneous pathogenesis of stroke. High numbers of CD19+CD86+ B cells correlated with more stenosis, whereas no correlations were found between CD19+CD40+ B cells and stenosis or carotid intima-media thickness. This may indicate that the CD19+CD40+ B cell subset does not influence development of carotid artery atherosclerosis but may modulate subsequent progression of atherosclerosis. Because the fraction and the absolute numbers of B cell subsets are 2 different measures, we decided to look at both in the context of CVD. The reason for the discrepancies between the fraction and the numbers and association with incident stroke has to be further evaluated. It may reflect that the fraction of the B cell subset in relation to other cells is important during some circumstances, whereas the absolute numbers are important during other.

The present findings that release of IL-10 from mononuclear cells correlated with the fraction of CD19+CD40+ B cells, whereas CD19+CD86+ B cells associate with a more proinflammatory cytokine profile, are in line with previous studies demonstrating the importance of CD40 for regulatory B cells and CD86 in the induction of Th1 cells. Interestingly, when mononuclear cells were stimulated with CD3/CD28-beads, only a few associations between the released cytokines and the fraction of the CD3+ T cells were detected. These findings indicate that B cells may have a more important role than T cells in regulating cytokine release. The association between CD19+CD40+ B cells and the release of IL-10 may reflect the regulatory properties of this B cell subset. The association between these cells and IL-8 requires further evaluation. Recently, it has been demonstrated in experimental studies that maturation of B cells into functional IL-10 producing effector cells needs not only CD40 interactions with T cells but also presence of IL-21. IL-21 was found to expand IL-10-producing regulatory B cells several fold, resulting in increased inhibition of experimental autoimmune disease, making analyses of this cytokine a target of great interest in future studies.

The antigen-presenting molecule HLA-DR is known to be upregulated on antigen-presenting cells after immune stimulation and CD40 stimulation has been shown to induce ICOSL on B cells. In accordance, ICOS has been demonstrated to have a critical role as a regulator of various immune responses, and accumulating evidence suggests that a dysregulation of the ICOS–ICOSL costimulatory pathway may lead to exaggerated autoimmune responses. The present study demonstrates that high surface expression of ICOSL on CD19+CD86+ B cells correlates with lower levels of released cytokines, suggesting a regulatory role of ICOSL, whereas a high surface expression of HLA-DR on both CD19+CD40+ and CD19+CD86+ B cells associates with a proinflammatory cytokine profile. In contrast, we found that high surface expression of HLA-DR on both B cell subsets was associated with less common carotid intima-media thickness, a finding that remains to be further elucidated.

There are some limitations of the present study that should be considered. Most importantly, our analyses were performed on cells that had been stored at –140°C for several years. As compared with initiating new prospective studies, this has the obvious advantage of allowing the studies to be completed within a relatively short period of time. However, it remains to be fully established how well thawed cells are representative of the original cell population. Although we were unable to detect any loss of cells when comparing cell numbers at freezing and thawing, we cannot exclude the possibility of a selective loss of B cells. Moreover, the same fluorochrome was used to identify CD14- and CD19-expressing cells, and B cells were gated based on their characteristic low side scatter. Experiments using different
fluorochromes for CD14 and CD19 revealed that ≥6% of the CD40- and CD86-expressing cells in the low side scatter gate were monocytes rather than B cells, and it is important to keep this limitation in mind when interpreting the present findings.

In summary, the novel findings in this study suggest that different B cell subsets may have diverse effects on the incidence of stroke. Our observations are in line with previous studies indicating that interactions of the costimulatory molecules CD40 and CD86 on B cells may have distinct functions in the immune responses. However, the impact of B cell subsets in CVD needs to be further evaluated in larger cohorts and also include more surface marker analysis.

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Disclosures
None.

References
In the development of atherosclerosis, the action of T cells has been intensely studied, whereas the possible role of B cells has received less attention, making them an interesting target for further studies. The present study provides evidence for an involvement of B cells in the incidence of stroke and suggests that both pathogenic and protective B cell subsets exist.

**Significance**
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Material and Methods

Study population
The Malmö Diet and Cancer Study (MDCS) is a prospective cohort (n=28449) study examining the association between diet and cancer. Subjects born between 1926 and 1945 and living in Malmö were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a sub-study focusing on cardiovascular risk (MDCS cardiovascular cohort, n=6103). In the present study we randomly selected 700 subjects participating in the cardiovascular cohort of MDCS, aged 63 to 68 years old (mean age 65). Participants were followed from baseline examination until first event of CVD, emigration from Sweden or death, up until December 31st, 2008. Ascertainment of cases and validity of the registries used (the Swedish Discharge Registry, the Stroke Register of Malmö and the Cause of Death Registry of Sweden) have been proven to be high. A CVD event was defined as a fatal or nonfatal MI (i.e. ICD-9: 410), fatal- or non-fatal stroke (ICD-9: 430, 431, 434 and 436), or death attributable to underlying CHD (ICD-9: 410- 414), whichever came first. Throughout the follow-up period 150 incident first event CVD cases (84 coronary events and 66 strokes) were identified. Hypertension was defined as blood pressure ≥140/90 mmHg or blood pressure lowering medication, high cholesterol as >5 mmol/l, smoking as current smoking. Blood pressure, body mass index (BMI), cholesterol, smoking and lipid levels were determined as previously described. One subject was excluded due to incomplete clinical data. The study was approved by the Regional Ethical Review Board in Lund and was conducted in accordance with the Helsinki Declaration. All subjects 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. Briefly, the right carotid bifurcation was scanned within a predefined window comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid arteries. All images for measurement of IMT, plaque thickness were obtained in the longitudinal projection showing the thickest intima-media complex. Plaque was defined as a focal thickening of the IMT exceeding 1.2 mm. The thickness of the common carotid intima–media complex, i.e. the mean distance between the leading edges of the lumen–intima and the media–adventitia interfaces of the far wall (mean IMT CCA), was measured off-line and along 1 cm section in the longitudinal projection using a specially designed computer-assisted image analyzing system based on automated detection of the echo structures, but with the option to make manual corrections by the operator. The maximum thickness of the intima– media (max IMT bifurcation) in the far wall of the carotid bifurcation was also measured off-line. Analyses of carotid stenosis was performed as described.

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, 12 min at room temperature. The cell 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 10 min, at room temperature). The cells were resuspended...
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 until analysis.

**Staining of mononuclear leukocytes and flow cytometry**

The cells were thawed, washed with preheated (37°C) PBS supplemented with 1% human serum and centrifuged at 330×g, 10 min at room temperature. The cells were then resuspended in complete RPMI 1640 media (containing 10% human serum, 1 mmol/L sodium pyruvate, 10 mmol/L Hapes, 50 U penicillin, 50 µg/mL streptomycin, 0.05 mmol/L β-mercaptoethanol and 2 mmol/L L-glutamine, GIBCO). From each subject included in the study 400 000 cells were stained with fluorochrome-conjugated antibodies for analysis of B cells. The antibodies used in this study were FITC-anti-CD19, FITC-anti-CD14, PerCP-biotin-anti-CD3, PerCP-biotin-anti-CD56, APC-anti-CD40, PB-anti-CD86, AF700-HLA-DR and PE-B7-H2 (ICOSL) all from Biolegend. The viability stain 7-Aminoactinomycin D (7AAD; Biolegend) was added to detect dead or dying cells. Stained cells were fixed in 1% paraformaldehyde and measured on a CyAn ADP flow cytometer (Beckman Coulter). The analysis was performed with FlowJo7.6 software (Tree Star). The same FITC-channel was used for the CD14+ and CD19+ cells and the lymphocytes were gated based on forward and side scatter parameters. Alive B cells identified as 7AAD-, CD3-, CD56- and CD19+ cells were further gated based on their expression of CD40 or CD86 (Figure 1). Measurements of insufficient technical quality (~5%) were not included in the statistical analyses. Used gating strategy excluded about 0.43% CD19+ cells within the high side scatter channel and included 0.82% CD14+ cells in the low side scatter channel. CompBeads (BD) were used to correct for fluorescence spillover in multicolor analyses and gate boundaries were set by fluorescence-minus-one (FMO) controls. To obtain absolute B cell numbers, the B cell subsets were expressed as percentages of the lymphocyte gate in the flow cytometric analysis and multiplied by lymphocyte numbers from the blood cell count analysis (System K-1000 with data unit DA 1000, TOA Medical Electronics Co). Mean Fluorescence Intensities (MFI) were normalized to the fluorescence of AccuCount particles (Spherotech Inc.), included as an internal reference in each sample.

**Analysis of cytokines in cell supernatants**

Mononuclear cells (4×10^5 cells/cell culture well) were cultured in complete RPMI and stimulated with CD3/CD28 beads (1 bead per 2 cells; Miltenyi Biotec) for 72 hours at 37°C in a cell incubator (5% CO₂). Thereafter, the cell supernatants were stored at -80°C until analysis. The concentrations of released cytokines were determined with multiplex technology (MesoScale Discovery).

**Statistics**

An independent sample t test was used to assess normally distributed continuous variables and a Chi-square test for proportions between subjects with and without a cardiovascular event. Non-parametric test (Mann-Whitney) was used to assess non-normally distributed continuous variables between cases and controls. Skewed variables were logarithmically transformed and Pearson correlation coefficient was used to examine the relationship among continuous variables as appropriate. A linear regression model was used to calculate independent associations. The Kaplan-Meier method was used to evaluate rates of cardiovascular event-free survival of B cell
subset tertiles, and differences were analyzed by log rank test. Cox proportional hazard regression models were used to compare incidence of cardiovascular events between B cell subset tertiles and to calculate risk factor adjusted hazard ratios (95% confidence interval, CI). Plots of the hazard function in different groups over time did not indicate that the proportional-hazards assumption was violated.

References