Activation of T Lymphocytes in Atherosclerotic Plaques

Jean-Charles Grivel, Oxana Ivanova, Natalia Pinegina, Paul S. Blank, Alexander Shpektor, Leonid B. Margolis, Elena Vasilieva

Objective—To decipher the immunologic mechanisms of plaque maturation and rupture, it is necessary to analyze the phenotypes and distribution of individual lymphocytes that migrate to the plaques, as well as their activation at different stages of plaque formation.

Methods and Results—We developed a protocol to isolate plaque-residing immune cells and analyze their status using polychromatic flow cytometry. We found that the composition and phenotype of T lymphocytes in the plaques differs from that in blood. CD4 and, in particular, CD8+ T cells in plaques are highly activated; the fraction of CD8 T cells coexpressing CD25 and human leukocyte antigen-D related in plaques was 6 times as large as in blood.

Conclusion—The first flow-cytosis analysis of individual T cells in atherosclerotic plaques indicates that plaques represent a separate immunologic compartment from blood with lymphocytes characterized by a high level of T-cell activation, which is compatible with the presence of antigen(s) that trigger infiltration activation of these cells. The ability to isolate and characterize these cells may lead to the identification of such antigens. (Arterioscler Thromb Vasc Biol. 2011;31:2929-2937.)

Key Words: atherosclerosis ■ blood cells ■ immune system ■ immunologic techniques
Table 1. Overall Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Patients (n=27)</th>
<th>Controls (n=7)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque source, n carotid/aorta</td>
<td>22/5</td>
<td>...</td>
<td>NS</td>
</tr>
<tr>
<td>Age, mean±SD, y</td>
<td>64±12</td>
<td>41±11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>20 (74)</td>
<td>3 (43)</td>
<td>NS (0.1276)MW</td>
</tr>
<tr>
<td>Degree of carotid artery stenosis, mean±SD, %</td>
<td>80.2±6.3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>3 (11)</td>
<td>1 (14)</td>
<td>NS (0.8487)MW</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>16 (59)</td>
<td>1 (14)</td>
<td>0.039 MW</td>
</tr>
<tr>
<td>Hypertension</td>
<td>23 (85)</td>
<td>1 (14)</td>
<td>0.0003 MW</td>
</tr>
<tr>
<td>Smoking</td>
<td>10 (37)</td>
<td>1 (14)</td>
<td>0.2699 MW</td>
</tr>
<tr>
<td>Previous ischemic history, n (%)</td>
<td>21 (78)</td>
<td>0 (0)</td>
<td>NS (0.0002 MW)</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>13 (48)</td>
<td>0 (0)</td>
<td>0.0228 MW</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>8 (30)</td>
<td>0 (0)</td>
<td>NS (0.1111 MW)</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>14 (52)</td>
<td>0 (0)</td>
<td>0.0154 MW</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>14 (52)</td>
<td>0 (0)</td>
<td>0.0154 MW</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>3 (11)</td>
<td>0 (0)</td>
<td>NS (0.3862 MW)</td>
</tr>
<tr>
<td>Statins</td>
<td>13 (48)</td>
<td>0 (0)</td>
<td>0.1157 MW</td>
</tr>
</tbody>
</table>

NS indicates nonsignificant; MW, Mann-Whitney test used.

The sample and was digested by an enzymatic mixture optimized as described in the Results.

Preparation of Peripheral Blood Mononuclear Cells

Patients’ peripheral blood, drawn with 3.8% Na citrate, was centrifuged for 10 minutes at 800g for 5 minutes, and the plasma was decanted, the red blood cells were lysed with a lysis solution (Biolegend, San Diego, CA) and centrifuged at 400g for 5 minutes, and the cells were resuspended in PBS.

Flow Cytometry

We determined the phenotypes of the mononuclear cells by flow-cytometric analysis on a FACSCanto II (BD Biosciences, San Jose, CA) equipped with 588-nm, 633-nm, and 405-nm LASER lines. For live/dead discrimination, cells were stained by adding 2 μL of a 1 mg/mL solution of the amine reactive dye Pacific Orange (succinimidyl ester triethlylammonium salt; Invitrogen, Carlsbad, CA) in dimethyl sulfoxide to 1 mL of cell suspension diluted in PBS. The stainings were performed on 100 μL of cell suspension, and the totality of the staining tube was acquired and analyzed. This allows the estimation of the number of cells contained within these 100 μL. In addition, for 6 plaques we estimated the number of cells per mg of tissue. Blocks of tissues from which the cells were to be isolated were weighted, and then the numbers of extracted cells were evaluated on the flow cytometer using the whole stained tube.

The live/dead staining was allowed to proceed for 15 minutes at room temperature; then, the cells were diluted in a larger volume of PBS supplemented with 2% normal mouse serum and, after centrifugation, resuspended in 1 mL of staining buffer.

Fifty microliters of cell suspension was stained with the following mixtures of monoclonal antibodies diluted at their optimal titer: (1) CD45 Cy7-phycocerythrin (PE), CD3 Cy5.5-PerCp, CD4 eFluor780-allophycocyanin (APC), CD28 PE, CD27 fluorescein isothiocyanate (FITC), CD197 APC, and CD45RA eFluor450 for the classification of memory and naïve T-cell subsets; (2) CD45 Cy7-PE, CD3 Cy5.5-PerCp, CD4 eFluor780-APC, CD28 PE, CD27 FITC, CD38 APC, and human leukocyte antigen-D related (HLA-DR) Pacific blue for the determination of T-cell activation; and finally, (3) CD45 Cy7-PE, CD3 Cy5.5-PerCp, CD4 eFluor780-APC, CD197 PE, CD16 FITC, CD25 APC, and CD8 eFluor450 for the analysis of T cells, B cells, and natural killer (NK) cells (Table 2). Not every sample was stained with all the above-listed markers.

In addition, cells were stained for, interferon (IFN)-γ, interleukin (IL)-2, IL-12, IL-22, IL-17A, T-Bet, and Foxp3. In the following

Table 2. Marker Used to Define Cell Subsets Assessed in This Study

<table>
<thead>
<tr>
<th>Subset</th>
<th>Low SSC</th>
<th>High CD45</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cell</td>
<td>CD45</td>
<td>(CD3+)*</td>
<td>CD19</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD45</td>
<td>(CD3+)*</td>
<td>CD16 CD56</td>
</tr>
<tr>
<td>NK T cells</td>
<td>CD45</td>
<td>CD3</td>
<td>CD16 CD56</td>
</tr>
<tr>
<td>T cells</td>
<td>CD45</td>
<td>CD3</td>
<td>(CD16+)* (CD56+)*</td>
</tr>
<tr>
<td>T-cell subsets</td>
<td>CD4</td>
<td>CD4+</td>
<td>(CD8+)*</td>
</tr>
<tr>
<td>Naïves</td>
<td></td>
<td></td>
<td>CD19 CD45RA+ CD27+ CD28+</td>
</tr>
<tr>
<td>Tcm</td>
<td></td>
<td></td>
<td>CD19+ (CD45RA-)* CD27+ CD28+</td>
</tr>
<tr>
<td>Terh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early differentiated</td>
<td>(CD19+)*</td>
<td>CD45RA-/-</td>
<td>CD27+ CD28+</td>
</tr>
<tr>
<td>Intermediately differentiated</td>
<td>(CD19+)*</td>
<td>CD45RA-/-</td>
<td>CD27+/- CD28+</td>
</tr>
<tr>
<td>Late differentiated</td>
<td>(CD19+)*</td>
<td>CD45RA-/-</td>
<td>CD27-/- CD28-</td>
</tr>
<tr>
<td>Treg</td>
<td>CD4</td>
<td>CD25</td>
<td>Foxp3</td>
</tr>
<tr>
<td>Th1</td>
<td>(CD8+)*</td>
<td>CD161</td>
<td>IL-17α IL-22</td>
</tr>
<tr>
<td>TH1</td>
<td>(CD8+)*</td>
<td>T-Bet</td>
<td>IFN-γ IL-2 IL-2</td>
</tr>
<tr>
<td>Activation markers</td>
<td>CD25</td>
<td>CD38</td>
<td>HLA-DR</td>
</tr>
</tbody>
</table>

NK indicates natural killer; SSC, side scatter; IL, interleukin; Tcm, central memory cells; Tem, effector memory cells; Treg, T-regulatory cell; TH, T helper; IFN, interferon; HLA-DR, human leukocyte antigen D-related.

*Exclusion marker: ie, the expression of this marker was used to gate out unwanted phenotypes.
†The exclusion of CD8 expression within T cells was used to define CD4 cells when this marker could not be included in the panel.
antibody combinations: (1) CD45 Cy7-PE, CD3 Cy5.5-PerCp, CD4 eFluor780-APC, CD25 PE, CD27 FITC, Foxp3 APC, and CD8 eFluor450 for detecting T-regulatory cells; (2) IFN-γ Cy7-PE, IL-22 Alexa Fluor 710-PE Cy5.5-PerCp, CD45 eFluor780-APC, CD161 PE, CD3 FITC, IL-17A APC, and CD8 eFluor450 for detecting Th17 cells; and (3) IFN-γ Cy7-PE, CD3 Cy5.5-PerCp, CD45 eFluor780-APC, T-Bei PE, IL-2 FITC, IL-12 APC, and CD8 eFluor450 for detecting Th1 cells. Cells were treated for 4 hours with brefeldin A in the absence of the cellular activators phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μmol/L) (Sigma-Aldrich). Stainings were performed using the fixation and permeabilization buffer provided with the FOX-p3 antibody (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Briefly, after cell surface staining, the cells were washed twice in staining buffer, resuspended in 1 mL of FOXp3 fixation/permeabilization buffer for 30 minutes at room temperature, and washed twice with flow cytometry staining buffer, and the pellet was resuspended in 100 μL of permeabilization buffer. Fifty microliters of intracellular antibody mix, diluted in staining buffer supplemented with 15% normal mouse serum and 5% normal rat serum, was added to each tube for 30 minutes at room temperature. The tubes were washed twice and fixed as described for cell surface staining. See Table 2 for marker selection.

We set up single-color compensation tubes for each staining in each experiment using Invitrogen’s Abc beads or Becton Dickin- son’s Comp beads. After staining, cells and beads were washed and fixed in 1% formaldehyde in PBS. The antibodies to CD14, CD146, CD16, CD19, CD197, CD3, CD38, CD4, CD45, CD56, and HLA-DR were purchased from Biolegend. CD16, CD25, CD27, CD28, CD3 cy5.5-PerCp, CD4, CD8, CD25, CD4, CD45RA, and CD8 were purchased from eBioscience; CD56 APC was purchased from Becton Dickinson. To position the analysis gates properly and to verify the specificity of the staining, for each staining, we performed the corresponding fluorescence minus 1 control set.5,6 These controls, in which 1 labeled antibody at a time is omitted systematically from the stain mix, allow the proper positioning of gates that take into account the complex spectral overlap observed in polychromatic cytometry. Also, for the intracellular staining for cytokines and nuclear transcriptional factor, we performed isotype control staining in addition to fluorescence minus 1 control.

Data acquisition was performed within 24 hours of staining and acquired by means of Diva 6.1.3. We analyzed data using FlowJo, version 9.3.3 (Tree Star, Ashland, OR).

Optimization of Enzymatic Digestion Mixture

To determine the optimal enzyme cocktail and concentration that would least affect the expression of cell surface markers, we digested fresh human peripheral blood mononuclear cells (PBMCs) isolated from normal volunteers’ PBMCs for 1 hour at 37°C with collagenase XIs (Sigma-Aldrich), collagenase IV (Invitrogen), or Liberase DL (Roche Diagnostics, Indianapolis, IN) in the presence of DNase I at 0.2 mg/mL (Roche Diagnostics). The cells were then harvested, washed, and stained with monoclonal antibodies according to the protocol outlined above. The levels of expression of the cell surface markers CD3, CD4, CD16, CD45, CD45RA, CCR7 CD28, CD27, HLA-DR, and CD38 in mock-digested PBMCs and control untreated PBMCs were compared.

All the blocks obtained from each plaque, at the exception of a few blocks frozen for future molecular analysis or for verifying the efficiency of digestion, were randomly distributed among 1.5-mL microcentrifuge digestion tubes (10 blocks per tube). Typically, for each plaque sample, 2 to 10 tubes were collected. Plaque fragments were digested for 1 hour at 37°C in a final volume of 0.5 mL of RPMI/10 blocks supplemented with the proper enzyme dilution (see Results) and 0.2 mg/mL DNase I. The released cells and tissue ghosts was strained through a 100-μm Celltrics filter (Partec, Münster, Germany), centrifuged at 400g for 8 minutes, resuspended in 2 mL of PBS, and processed for flow-cytometric analysis as described above. At the end of the digestion, all cells from the tubes were pooled and processed for staining.

To evaluate the efficiency of cell liberation from the plaques using the protocol described above, we cryosectioned several plaque tissues before and after lymphocytes liberation. We found virtually no lymphocytes in the digested samples (less than 4% of what was present before the digestion). Thus, our protocol liberates the majority of lymphocytes and their subsets.

Statistical Analysis

All the data obtained in the present work were normally distributed as assessed by the D’Agostino and Pearson omnibus normality test. The variance was evaluated for homoscedasticity using the Levene, Bartlett, and F tests.

We tested the null hypothesis that there was no difference between the mean values for plaque and blood of each dependant variable, the percentage of cells with a given antigen expression. t-tests were used to compare variables between plaque and blood and between male and female patients. If the data distribution was not homoscedastic as measured by F-test, the Welch’s correction for the t-test was used. When comparing data across multiple groups, ie, patient blood, patient plaque, and normal blood, an ANOVA was performed using the Tukey-Kramer honestly significant difference correction for multiple comparisons. When the data were not normally distributed, we used the Wilcoxon rank score test (Mann-Whitney) for 2-group comparisons and the Kruskal-Wallis rank sum test for comparison across several groups. Statistical analysis was performed using Prism 5.0d (GraphPad Software, La Jolla, CA) and JMP 9.0 (SAS Institute Inc, Cary, NC). Statistical significance was defined at an α level of 0.05.

Results

Development of a Digestion Protocol for Atherosclerotic Plaques

To separate individual cells from the plaques, we first used various collagenases that were first tested on PBMCs for preserving the cell surface markers CD3, CD4, CD16, CD45, CD45RA, CD197, CD28, CD27, HLA-DR, and CD38. We treated PBMCs with collagenase XIs diluted at 5, 10, and 2.5 mg/mL. The lowest enzyme concentration resulted in the reduction of CD8 expression by 90% compared with non-treated cells. Also, the expression of CD4, CD3, CD27, and CD28 was reduced by 40%, 12%, 50%, and 42%, respectively. Because of these strong effects, the ability of this enzyme to liberate cells was not studied further.

Second, we treated PBMCs with collagenase IV at 10, 5, and 2.5 mg/mL and noticed that at the highest concentrations, the expression of CD4 and CD8 were reduced by 29% and 23%, respectively. At 1.25 mg/mL collagenase IV, the surface markers expression was decreased by 4.9±3.4% compared with that on untreated PBMCs.

Third, we treated cells with Liberase DL. At the concentrations of 62.5 and 31.5 μg/mL, CD4 was reduced by approximately 70%, whereas at 15 μg/mL, the expression of this marker was reduced by only 20±7%. The expression levels of CD3, CD8, CD16, CD45, and HLA-DR were affected marginally.

To liberate cells from atherosclerotic plaques, we applied enzymes at concentrations that did not grossly decrease expression of cell surface molecules in PBMCs. Freshly excised plaques were digested with collagenase IV (2.5 and 1.25 mg/mL) and Liberase DL (25 and 12.5 μg/mL) in the presence of 0.2 mg/mL DNase I for 1 hour at 37°C. The cells were then stained with monoclonal antibodies for CD45, CD3, CD4, and CD8. Collagenase IV at 1.25 mg/mL liberated more lymphocytes than did digestion with 2.5 mg/mL as
evaluated by the relative amount of lymphocytes in the gate defined on side scatter (SSC) versus CD45 expression.

On the basis of the relative lymphocyte (CD45+) yield, we chose treatment with collagenase IV for 1 hour at 37°C as an efficient method to liberate lymphocytes from atherosclerotic plaques while largely preserving cell surface markers. We used this protocol in all our subsequent experiments described below. However, the concentration of the enzyme has to be adjusted for each enzyme batch and in our experiments varied between 1 and 1.25 mg/mL.

Comparison of the Main Lymphocyte Subsets Isolated From Plaques and Blood

We stained cells for a set of markers whose combination permits the classification of lymphocytes into CD4 T cells, CD8 T cells, B cells, and NK cells using the gating strategy depicted in Figure 1. Lymphocytes were defined as the lowest SSC population with high CD45 expression, monocytes as the cells having an intermediate SSC with high CD45 expression, and granulocytes as cells with a very high SSC and a lower expression of CD45. T cells were identified from their expression of CD3, CD4, and CD8 and divided into naïve (CD45RA+CD28+), central memory (CD45RA−CD28+), effector memory (CD45RA−CD28−), and memory cells, followed by the division into central memory cells (Tcm) (CD45+CD3+CD45RA−CD197+CD27+CD28+), and effector memory cells (Tem) (CD45+CD3+CD45RA−/−CD197−). Analysis of these narrowly defined subpopulations of T cells revealed significant differences between blood and plaque.

Subpopulations of CD8 T Cells Differ Between Plaque and Blood

Among CD8 T cells, the fraction of Tcm differed between plaque and blood (18.25±1.84% and 13.16±1.53% of CD8 T cells, respectively; n=28 and n=25, P=0.042). Similarly, Tem constituted a lower fraction of CD8 T cells in plaques than in blood (Figure 3A), representing 64.96±2.50% (n=28) and 76.23±2.82 (n=25; P=0.0042), respectively.

On the basis of the expression of CD28 and CD27, Tem cells can be subdivided into early-differentiated (CD45+CD3+CD45RA−/−CD197−CD27+CD28+) (Tem ED), intermediate (CD45+CD3+CD45RA−/−CD197−CD27+/−CD28+) (Tem ID), and late-differentiated cells (CD45+CD3+CD45RA+/−CD197−CD27−CD28−) (Tem LD). CD8 Tem distribution among these 3 maturation subsets was different in plaques and blood from the plaque donors (Figure 3B). The fraction CD8 Tem ID cells represented 39.20±2.87% and 22.25±2.74% of CD8 Tem cells (P<0.001) in plaque and blood respectively. The fraction CD8 Tem (LD) represented 29.25±3.61% and 53.45±4.98% of CD8 Tem in plaque (n=28) and in patient blood (n=25; P<0.001), respectively. In contrast, the fractions of CD8 Tem ED cells in plaque and blood were not different and represented 31.5±2.96% in plaque (n=28) and 24.28±3.71% in patients’ blood (n=25; P=0.13).

Also, naïve CD8 T cells were unequally distributed between plaques and blood, and represented 2.86±0.73% and 8.13±2.0% (n=28 and n=25, P=0.019), respectively, of CD8 T cells in these tissues.

CD4 T Cell Subpopulations in Plaques and Blood

Unlike CD8 T cells, CD4 Tcm cells were less represented in plaques than in blood, making up 27.68±2.43% and 50.44±2.99%, respectively, of CD4 T cells in these compartments (n=28 and n=25, P<0.001) (Figure 3A). On average, CD4 T cells in plaques were more differentiated than those in blood, with Tem making up 39.95±2.73% and 23.79±2.27%, respectively, of CD4 T cells in these tissues (n=28 and n=25, P<0.001) (Figure 3B). CD4 Tem ED were equally represented in plaques and in blood, and made up 29.06±2.68% and 32.0±3.11%, respectively, of CD4 Tem (n=28 and n=25, P=0.476). CD4 Tem ID cells represented 60.24±3.1% and 37.38±3.34% of CD4 Tem in plaques and blood, respectively (n=28 and n=25, P<0.001), and finally, CD4 Tem TD cells represented 10.68±3.25% and 30.61±4.18% of CD4 Tem (n=28 and n=25, P<0.001) in plaques and blood, respectively. Naïve CD4 T cells were unequally distributed between plaque and blood and represented 3.34±0.73% and 19.27±3.21% (n=28 and n=25, P<0.001), respectively, of CD4 T cells in these tissues. Thus, the T-cell spectra are different in peripheral blood and atherosclerotic plaques.

Also, we performed intracellular staining for T-regulatory cells and compared their fractions in plaque and blood. We
Figure 1. Gating strategy for lymphocyte analysis. Single cells were identified on a forward scatter area (FSC-A) vs their forward scatter height (FSC-H) bivariate plot, and dead cells were excluded by their positive staining with an amino-reactive “live-dead” dye. Lymphocytes were defined as low side scatter (SSC) cells brightly stained with CD45 (A). Among lymphocytes, T cells were identified by their expression of CD3 (B) and were divided into CD4⁺/H11001 and CD8⁺/H11001 (CD4⁺/H11002) in a bivariate plot of CD3 vs CD4 (C). Based on their expression of CD197 and CD45RA (D), each T-cell fraction (CD4⁺/H11001 and CD4⁺/H11002) was further classified into naïve cells (CD45RA⁺/H11001 CD197⁺/H11001), central memory cells (Tcm: CD197⁺/H11001 CD45RA⁺/H11002), and effector memory cells (Tem: CD45RA⁺/H11001 CD197⁻). Each subpopulation was then finally analyzed for the expression of CD28 and CD27 to refine the phenotype of naïve cells (CD45RA⁺/H11001 CD197⁺/H11001 CD27⁺/H11001 CD28⁺/H11001) (E) and to divide Tem into early (CD27⁺/H11001 CD28⁺/H11001), intermediate (CD27⁺/H11001 CD28⁻/H11001 CD27⁺/H11001 CD28⁻/H11001), and late differentiated (CD27⁻/H11001 CD28⁻/H11001) (F).
NK T cells. discontinuous to better visualize the low abundance of B and lymphocytes defined as described in Figure 1A–1C. The and their relative abundance is expressed as percentage of lym-

identified by their expression of CD3, CD19, and CD56 CD16, and the relative abundance is expressed as percentage of lym-

phocytes as defined in Figure 1A–1C. The y-axis is discontinuous to better visualize the low abundance of B and NK T cells. B, Comparison of the relative abundance of CD4 and CD8 T cells, excluding NK cells. The probability values represent the results of paired t-tests. *0.01<P<0.05, **P<0.01.

defined T-regulatory cells as CD4 T cells that expressed the transcription factor Foxp3 and the IL-2 receptor CD25. Cells isolated from the plaques and blood of 5 patients were stained for T cell surface markers and CD25, fixed, and permeabil-

ized with a Foxp3 compatible buffer system. We did not find any difference between plaque and blood regarding the presence of T-regulatory cells. In plaques, Foxp3 CD25+ cells represented 2.29±0.89% of CD4 T cells, whereas in blood they represented 0.95±0.53% of CD4 T cells (P=0.313).

Finally, because the availability of specimens obtained from men and women was different, we investigated whether the predominance of male patients in our cohort could affect the results. We compared T cell subsets in plaques and blood from 7 women with those from 20 men. We found no statistically significant differences in the frequencies of B cells (P=0.48), NK cells (P=0.345), T cells (P=0.667), CD4 T cells (P=0.370), and CD8 T cells (P=0.312) from patients of different genders. Neither there were differences in CD8 T cell subsets in any subpopulations analyzed at the exception of CD8 Tcm and CD8 Tem. The fraction of CD8 Tcm was slightly lower in women than in men (10.11±1.6% versus 17.53±1.45%, P=0.012).

Fraction of CD8 Tem was higher in women than in men (78.69±2.79% versus 67.81±2.32%, P=0.022). Similarly, the fine analysis of CD4 T cell phenotypes showed that the only difference between men and women was in the proportion of CD28−CD4+ T cells, which were higher in women (11.95±2.84 versus 6.53±1.01%, P=0.033).

Analysis of the Activation Status of T Cells in Plaque and Blood

We compared the expression of the late activation markers CD25, CD38, and HLA-DR, as well as of IFN-γ and IL-2 on CD8 and CD4 T cells isolated from plaques and from the blood of the individuals from whom these plaques were obtained.

Activation of CD8 T Cells in Plaques and Blood

Analysis of the expression of these activation markers revealed that in plaques, CD8 T cells are more activated than in blood (Figure 4A). CD25 was expressed by 23.05±4.6% (n=28) and 7.41±4.77% (n=26; P=0.02) of CD8 T cells in plaques and blood, respectively. CD38 was also expressed on a larger fraction of CD8 T cells isolated from plaque than from blood: 41.26±2.69% (n=28) and 34.55±2.79%
IL-17 and CD161 (0.48±0.26% versus 0.08±0.04% of CD8 T cells, respectively, P=0.186).

Similarly, more plaque than blood CD4 T cells expressed CD161 on stimulation (63.16±2.28% versus 28.97±6.02%, respectively, n=5, P=0.003) and secreted IL-17 (1.5±0.48% versus 0.25±0.13%, n=5, P=0.041). However, when these 2 markers we combined to identify Th17 cells, the differences vanished, and CD161,Th17 cells represented 0.91±0.42% versus 0.13±0.17% of CD4 T cells in plaque and blood, respectively (P=0.12). Thus we found no statistical difference in cytokines’ expression between artificially stimulated T cells from blood and from plaques, probably because of the large variation of the data.

In the above-presented analysis, we compared fractions of T cells and status in plaques and blood from the same patients. Here, we compared these parameters with those of cells from 7 healthy donors using an ANOVA across these 3 groups and the post hoc analysis with the Tukey-Kramer honestly significant difference correction for multiple comparisons. The difference between T-cell fractions of lymphocytes (defined by their expression of CD45 in a low SSC gate) in these 3 tissues was significant (P=0.026). However, the only significant difference was between the fraction of T cells in plaque and patient blood (P=0.008). A significant difference was revealed between the 3 groups in the fractions of CD4 and CD8 T cells (P<0.001 and P=0.007, respectively). In addition to the above-described differences between plaque and patients’ blood, the post hoc analysis revealed differences between plaque and control blood in the fraction of CD4 (P=0.002) and CD8 (P=0.028) T cells. In contrast, the CD4 and CD8 fractions were not different between plaque donors’ blood and control blood (0.7<P<0.77). The analysis of CD4 T cells subsets confirmed all the above-described differences between plaque and patient blood, but it also revealed differences between patients’ blood and control blood: the latter contained significantly more CD4 Temo ED cells than patients’ blood (51.3±5.6% versus 32.0±2.97, P=0.0098), whereas CD4 Tem LD cells were more abundant in patients’ blood than in control blood (30.6±3.65% versus 9.3±6.9%, P=0.023). Naïve CD4 T cells were found to be more abundant in control blood group than in patients’ blood (40.18±4.26% versus 19.27±2.25%, P<0.001). Finally this analysis revealed a difference in the distribution of CD4+CD28– T cells, which was only significant between patients’ blood and control blood (9.16±1.39% versus 1.91±2.5%, P=0.043).

The analysis of the CD8 T cell subsets in the 3 groups revealed that blood from control donors contained significantly less CD8 Tem than patients’ blood (48.97±5.49 versus 76.23±2.91% P<0.001). Although the ANOVA revealed differences in the distribution of CD8 Tem ID and CD8 Tem LD between the 3 tissues (P=0.0001 and P=0.0006 respectively), the post hoc analysis did not show any difference between patients’ blood and control blood (0.143<P<0.9359). Naïve CD8 T cells were not equally distributed among the 3 groups (P<0.001); however, the post hoc analysis did reveal a difference between patients’ blood and plaque and significant differences between control blood (33.40±3.18%) and patients’ blood (P<0.001) and plaques (P<0.001).
The fractions of B cells and NK cells were not significantly different when compared in plaques, blood from the plaques’ donors, and normal blood \((P=0.058\text{ and } P=0.11\) respectively).

In summary, our data show that plaques are highly enriched with activated T cells, especially of the CD8 phenotype.

**Discussion**

Although since Rudolph Virchow’s time it has been suspected that inflammation contributes to the development of atherosclerosis, its pivotal role in the growth and rupture of plaques began to be appreciated only recently. The amounts of many inflammatory cytokines strongly correlate with the mortality of coronary artery disease patients.\(^1,10\) Also, an increase in the fraction of particular blood lymphocyte subsets has been reported to be associated with higher frequency of cardiac events in patients with coronary artery disease, rheumatoid arthritis, diabetes mellitus, and kidney insufficiency.\(^11-14\) Not only systemic but also local inflammation, even distant inflammation, affects the status of atherosclerotic plaques, as for example evidenced by a significant association between periodontitis and coronary atherosclerosis.\(^15\) Even more importantly, inflammation at the site of the plaque may affect plaque growth and rupture, resulting in clinical events such as unstable angina or acute myocardial infarction. Like other cases of inflammation, inflammation at the site of a plaque should be manifested by the infiltration and activation of the cells of the immune system.\(^3,16,17\)

However, a comprehensive single-cell analysis of individual cells in plaques has not yet been performed except with traditional histochimistry, which demonstrated that there are more lymphocytes\(^4,18\) and dendritic cells\(^19\) in complicated carotid endarterectomy specimens than in control arteries. One of the main obstacles to modern cutting-edge multiparameter characterization of plaque cells with multicolor flow cytometry stems from the difficulty in isolating cells from plaque tissue without impairing the expression of their surface markers, which define the cells’ identity. In this study, we overcame this problem and developed an original protocol for isolation of plaque lymphocytes that preserves their surface markers.

We tested different enzyme cocktails on PBMCs, where the surface markers of the treated samples could be compared with those of the untreated control. We chose an optimal enzyme cocktail combination based on collagenase IV, which liberates cells from atherosclerotic plaques while preserving their identity. This allowed us to perform a meaningful multicolored flow-cytometric analysis of these cells.

The first question we asked was whether plaques represent a separate immunologic compartment with lymphocytes different from those in blood. We found that indeed, lymphocytes in plaques and blood differed significantly: plaques were enriched with CD8 T cells (the average CD4/CD8 ratio in blood was 2.76±0.4, whereas in plaques it was only 1.3±0.24). Furthermore, T lymphocytes, especially CD8 T cells in plaques, were more activated than in blood, as evaluated from the expression of the CD25, CD38, and HLA-DR activation markers. The fraction of cells coexpressing CD25 and HLA-DR in plaques was 6 times as large as in blood. Although in plaques, the fractions of T cells expressing activation markers were high, at the time of isolation, the frequency of cells spontaneously producing cytokines was below our level of detection unless the cells were artificially activated after isolation. We observed a similar phenomenon for blood cells, in agreement with what was reported earlier for unstimulated blood\(^20-22\) and with the common practice of using unstimulated blood as negative control in intracellular cytokine staining. Short of knowing the antigens responsible for the activation of these cells in vivo, only ubiquitous polyclonal activators can reveal the pattern of T cell cytokine polarization, which happened in vivo; phorbol 12-myristate 13-acetate is such an activator.\(^20\)

In general, our data indicate that plaque lymphocytes were not contaminants of our preparation and did not penetrate plaques indiscriminately. Moreover, CD8 T cells in plaques not only were more abundant but also were more activated than in blood, indicating a local inflammatory-like process that takes place in plaques. One possible explanation of the fact that CD8 rather than CD4 T cells seemed to be activated is that the antigens that activate T cells may be of viral or endogenous nature (eg, a heat shock protein or oxidized forms of LDL.\(^3,11,23,24\) In the future, our approach may allow the expansion of T cells to test this speculation.

One of the drawbacks of our current study is that we did not differentiate between different types of plaques. Reliable differentiation of plaques into ruptured and nonruptured is complicated by possible tissue damage during atherectomy. However, the use of our protocol for T cell isolation in future studies will allow us to analyze possible differences between the T lymphocytes in tissues of individuals with ruptured and nonruptured plaques and plaques with superficial erosions.

Also, it would be important to follow, on a larger number of samples, the differences between men and women in plaques’ T cells. In particular, it would be interesting to extend our observation on the higher proportion of CD28\(^−\)CD4\(^+\) women’s plaques compared with men’s. The high proportion of these cells in blood is associated with recurrent coronary instability, type 2 diabetes mellitus,\(^14,22\) and polycystic ovary syndrome with high risk of acute coronary syndrome.\(^26\) The high proportion of CD28\(^−\)CD4\(^+\) in women’s plaques may be relevant to the lesser obstructive coronary disease in women with acute coronary syndrome compared with men.\(^27\) Another promising future work may focus on the analysis the T cell subpopulations in patients with superficial erosions of plaques that are more common in women.\(^28\)

Finally, in the current work we focused on comparing plaque and blood cells from the same donors. Another important comparison, namely between blood of patients with plaques and that of healthy individuals, was largely outside the scope of our work because it requires matching the 2 groups for many parameters. Comparing T cells isolated from plaque and blood of patient with cardiovascular disease overcomes this problem. Nevertheless, in the present work, we compared the cell composition of 3 groups of samples: plaque cells, blood cells of the plaques’ donors, and blood cells of healthy individuals. We found that blood obtained from patients with plaques contained more CD4 Tem, terminally differentiated CD4 T cells, and CD4\(^+\)CD28\(^−\) T cells but fewer naïve CD4 and CD8 T cells, as well as early differentiated CD4 Tem. Also, blood from plaque donors
contained more activated T cells. These results are in agreement with the earlier published works.3,11,23

In general, the enrichment of plaques with CD8 T cells and the preferential activation both of these cells and of CD4 T cells compared with activation levels in blood may indicate the presence of foreign antigens in these plaques, as was suggested earlier.4,18,24,29–31 Identification of antigens that have triggered T cells to migrate to the endothelial wall32 and activation in plaques requires analysis of the reaction of individual T cells to these antigens. With the development of the protocol of isolation and identification of T cells from plaques that is described in the present article, such analysis is becoming possible.

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Disclosures

None.

References


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