Expansion of T-Cell Receptor $\xi^{\text{dim}}$ Effector T Cells in Acute Coronary Syndromes

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Objective — The T-cell receptor zeta (TCR$\xi$)-chain is a master sensor and regulator of lymphocyte responses. Loss of TCR$\xi$-chain expression has been documented during infectious and inflammatory diseases and defines a population of effector T cells (TCR$^{\text{dim}}$ T cells) that migrate to inflamed tissues. We assessed the expression and functional correlates of circulating TCR$^{\text{dim}}$ T cells in coronary artery disease.

Methods and Results — We examined the expression of TCR$\xi$-chain by flow cytometry in 140 subjects. Increased peripheral blood CD4$^+$ TCR$^{\text{dim}}$ T cells were found in patients with acute coronary syndromes (ACS, n=66; median 5.3%, interquartile 2.6 to 9.1% of total CD4$^+$ T cells; $P<0.0001$) compared to chronic stable angina (CSA, n=32; 1.6%; 1.0 to 4.1%) and controls (n=42; 1.5%; 0.5 to 2.9%). Such increase was significantly greater in ACS patients with elevated levels of C-reactive protein, and it persisted after the acute event. Moreover, TCR$^{\text{dim}}$ cells were also more represented within CD8$^+$ T cell, NK, and CD4$^+$CD28null T cell subsets in ACS compared to CSA and controls. Finally, CD4$^+$ and CD8$^+$ TCR$^{\text{dim}}$ T cells isolated from ACS displayed an enhanced transendothelial migratory capacity.

Conclusions — TCR$^{\text{dim}}$ T cells, an effector T-cell subset with transendothelial migratory ability, are increased in ACS, and may be implicated in coronary instability. (Arterioscler Thromb Vasc Biol. 2008;28:2305-2311.)

Key Words: acute coronary syndrome ■ lymphocytes ■ flow cytometry ■ immune system ■ receptors

Inflammation plays a key role in the pathogenesis of atherosclerosis with participation of both innate and adaptive immunity.1–3 Uregulation of proinflammatory cytokines is common in acute coronary syndromes (ACS).4 Elevated C-reactive protein (CRP) levels are associated with an adverse prognosis in patients with coronary artery disease (CAD), and healthy subjects.4 The activation of inflammatory pathways in ACS is not confined to coronary lesions but involves the activation of neutrophils, monocytes, and lymphocytes in peripheral blood.5–7 Most T cells found in human atherosclerotic lesions exhibit an effector or memory phenotype with a Th1 bias, and a predominance of CD4$^+$ over CD8$^+$ T cells.8 Different subsets of T cells may drive or regulate inflammation during evolution of the atherosclerotic process, as in other inflammatory diseases.9 An increased prevalence of specific circulating T cell subsets, such as CD4$^+$CD28null T cells, was reported in patients with ACS, as well as in patients with rheumatoid arthritis.10–13

The TCR$\xi$-chain (CD247 or CD3$\xi$) is a transmembrane protein with a small extracellular domain and an intracellular domain containing 3 immunoreceptor tyrosine-based activation motifs. The TCR$\xi$-chain subunit associates with the TCR-CD3 complex as a homodimer, and couples antigen recognition by the TCR to downstream intracellular signal-transduction pathways through phosphorylation and recruitment of downstream proteins. The TCR$\xi$-chain is also expressed in NK cells, where it is associated with the FcR$\gamma$III low affinity IgG receptor (CD16) and other activating receptors.14 TCR$\xi$-chain is therefore a master regulator and sensor of innate as well as adaptive immune responses, and so it follows from this that aberrations in its expression or function should be expected to have profound effects on immune function.15

A subset of T cells expressing low levels of TCR$\xi$-chain (hereafter TCR$^{\text{dim}}$ T cells) has been described in association with infectious, malignant, and inflammatory diseases.14–18 Downregulation of the TCR$\xi$-chain, known to occur after antigen engagement or in response to inflammatory stimuli, may represent an attempt to modulate the immune response.14 However, we previously reported that the TCR$^{\text{dim}}$ T-cell subset, although refractory to TCR-induced proliferation,19
paradoxically displays features of antigen-experienced effector T cells.\textsuperscript{15} Cell surface markers and tetramer analysis revealed that TCR\textsubscript{dim} T-cells are enriched for memory CD45RO\textsuperscript{+} cells, and for cells that had previously engaged antigen. Moreover, TCR\textsubscript{dim} T cells produce high levels of interferon (IFN) \(\gamma\) and tumor necrosis factor (TNF) \(\alpha\), and low levels of interleukin (IL)-10.\textsuperscript{15} Finally, TCR\textsubscript{dim} T-cells display effector functions, e.g., they can activate monocytes via cell contact-dependent pathways, and preferentially accumulate in inflamed tissues such as the rheumatoid joint.\textsuperscript{15}

In the present study we report that the frequencies of subsets of T cells and NK cells expressing low levels of TCR\(z\)-chain are increased in patients with ACS when compared to patients with stable atherosclerotic lesions or to controls, particularly in patients with elevated levels of CRP, and remain elevated after the acute coronary event. TCR\textsubscript{dim} T cells display an enhanced ability to migrate through activated endothelium in vitro. Our findings suggest the possibility that antigen experienced T cells, defined by the TCR\textsubscript{dim} phenotype, may contribute to the inflammatory process that triggers coronary instability by accumulating in coronary plaques.

**Methods**

**Study Population**

Institutional Ethics Committees approved the study, and informed consent was obtained from all participating subjects. Venous peripheral blood samples were obtained from all patients on admission to San Raffaele Scientific Institute. Three groups participated in the study. Control group comprised 42 individuals with negative history, clinical, and electrocardiographic signs of CAD. CSA group included 32 patients with effort angina (lasting more than 3 months and without previous history of unstable angina or myocardial infarction) with angiographic evidence of coronary artery stenosis (stenosis >50% diameter). ACS group comprised 66 patients with chest pain accompanied by ischemic electrocardiographic changes (ST-segment changes or T-wave inversions) or ST-segment elevation >2 mm in at least 2 consecutive leads associated to increase of troponin I. All samples were obtained on admission. Evidence of coronary artery stenosis/occlusion was documented by coronary angiography. Exclusion criteria included recent surgery, documented immune, infectious or neoplastic disease, immunosuppressive therapy, ACS attributable to intrathrombus, or occlusion of arterious and venous by-pass grafts (confirmed after angiography). In 17 patients with ACS the levels of TCR\(z\)-chain were also assessed after a 50-day median time (interquartile ranges 39 to 83 days) from admission. Further analysis of TCR\(z\)-chain expression was performed in CD\(8^+\) T and NK cells (14 control, 9 CSA and 22 ACS participating in the study) and in CD\(4^+\)CD28\textsuperscript{null} T cells (19 controls, 18 CSA and 23 ACS).

**Cell Isolation**

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque (Becton Dickinson) density gradient centrifugation from anticoagulated venous blood samples, before flow cytometry. Alternatively, flow cytometry was performed on fresh whole blood performed using anti–CD3-Cascade Yellow (clone UCHT1, Dako), anti–CD3-PE-Cychrome 7 (clone SK3 Becton Dickinson), anti–CD28-FITC (clone HIT8a), anti–CD3-PE (clone 2H2D9, Immunotech, Coulter), anti–TCR\(z\)-FITC (cloneG3,Dako). The 2 different clones used of anti–TCR\(z\) individuate the same percentage of TCR\textsubscript{dim} cells. The CD3\(CD56^+\) NK cell subset was found to be uniformly TCR\(z\) negative, and was not therefore studied further.

**TCR\(z\) Expression Analysis**

Surface staining of T cell subsets was performed by standard methods. For intracellular staining, cells were fixed with 2% formaldehyde and permeabilized in buffer containing 10 \(\mu\)g/mL saponin. The efficiency of permeabilization was determined by uptake of trypan blue (>99% in all experiments). Isotype-matched controls Abs were used to confirm expression specificity. The following Abs were purchased from Becton Dickinson: mouse IgG1 isotype control conjugated with PE (clone MOPC-21) or with fluorescein isothiocyanate (FITC) (cloneA85-1), anti-CD3-FTC (clone UCHT1), anti–CD4-PE-Cychrome 5 (clone RPA-T4), anti–CD8-Cychrome 5 (clone HIT8a), anti–CD3-PE-Cychrome 5 (clone HIT3a), anti–CD16-FITC (clone 3G8), anti–CD3-Pacific Blue (PB) (clone UCHT1), anti–FoxP3-PB (clone 206D). Two different antibodies were used for TCR\(z\), expression studies: anti–TCR\(z\)-PE (clone 2H2D9, Immunotech, Coulter), anti–TCR\(z\)-FITC (cloneG3,Dako). The 2 different clones used of anti–TCR\(z\) individuate the same percentage of TCR\textsubscript{dim} cells. The CD3\(CD56^+\) NK cell subset was found to be uniformly TCR\(z\) negative, and was not therefore studied further.

We confirmed data also with fresh whole blood analysis (cell viability >99%). Cell viability was assessed using the Molecular Probes Patented LIVE/DEAD Viability (Invitrogen) according to the manufacturer instructions. Moreover, frequencies of TCR\textsubscript{dim} within the CD3\(CD4^+\)CD28\textsuperscript{null} subset were determined by a 4-color flow cytometry on fresh whole blood performed using anti–CD3-Cascade Yellow (clone UCHT1, Dako), anti–CD4-activated protein C (APC)-Cychrome 7 (clone SK3 Becton Dickinson), anti–CD28-FITC (clone 2CD28.2, Becton Dickinson), and anti–TCR\(z\)-PE (clone 2H2D9, Immunotech, Coulter).

Cells were analyzed on a Cyan ADP (Dako) flow cytometer. FCS Express version 3 (De Novo Software) was used for analysis. The percentage of TCR\textsubscript{dim} was determined within each lymphocyte subset of interest. In addition, we assessed the Median Fluorescent Intensity (MFI) of TCR\(z\)-chain expression as MFI index (MFI TCR\(z\)\textsubscript{high}/MFI TCR\(z\)\textsubscript{negative}) as described.\textsuperscript{18}

### Table. Clinical Characteristics and Biological Parameters of Patients and Healthy Individuals

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CSA</th>
<th>ACS</th>
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<tbody>
<tr>
<td>n</td>
<td>42</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>Age, means (±SD)</td>
<td>57 (±16)</td>
<td>65 (±11)</td>
<td>63 (±10)</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>33 (79)</td>
<td>27 (84)</td>
<td>55 (83)</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>20 (48)†</td>
<td>23 (72)</td>
<td>49 (74)</td>
</tr>
<tr>
<td>Smoking</td>
<td>3 (7)*</td>
<td>15 (47)</td>
<td>24 (36)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0 (0)</td>
<td>4 (13)</td>
<td>4 (6)</td>
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<tr>
<td>Hypercholesterolemia</td>
<td>14 (44)</td>
<td>14 (64)</td>
<td>27 (51)</td>
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<tr>
<td>Laboratory parameters, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell count, ×10(^9)</td>
<td>6.3 (5.6–7.9)*</td>
<td>7.2 (6.4–9.3)</td>
<td>9.2 (6.9–11.9)</td>
</tr>
<tr>
<td>Troponin I, ng/ml</td>
<td>0</td>
<td>0</td>
<td>0.4 (0.0–5.9)</td>
</tr>
<tr>
<td>Medication on admission, n (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-Blockers</td>
<td>2 (5)*</td>
<td>19 (59)†</td>
<td>19 (29)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3 (7)*</td>
<td>29 (93)*</td>
<td>32 (48)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>5 (12)†</td>
<td>13 (41)</td>
<td>17 (28)</td>
</tr>
<tr>
<td>Statins</td>
<td>2 (5)*</td>
<td>18 (56)*</td>
<td>12 (18)</td>
</tr>
</tbody>
</table>

\(\ast P<0.001; \dagger P<0.01; \ddagger P<0.05\) vs ACS.
Confocal Microscopy
Cells from 10 subjects randomly chosen among ACS patients (n=3), CSA patients (n=2) and controls (n=5) were fixed and stained for confocal microscopy with anti–CD3-PB (clone UCHT1, Becton Dickinson) or anti–CD3-FITC (clone UCHT1, Becton Dickinson) and, on permeabilization, anti–TCRζ-FITC (clone G3, Dako) or anti–TCRζ-PE (clone 2H2D9, Immunotech, Coulter). Cells were subsequently plated onto glass slides and examined under a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems). Z-series were collected from singles channels, processed to 2D free projection max images, and merged. Single stains either for CD3 and TCRζ served as controls.

Measurement of High Sensitivity CRP (hsCRP)
Peripheral blood samples were centrifuged and serum aliquots were stored at −80°C until assayed in single batch. hsCRP was assessed via nephelometry (BN II–Behring instrument).

Transendothelial Migration Assay
We performed transendothelial migration assays of T cells in 17 study subjects (5 controls, 6 CSA, and 6 ACS), by applying 10⁶ lymphocytes to gelatin-coated transwell upper chambers containing a monolayer of human umbilical vein endothelial cells (HUVECs) previously stimulated with 10 ng/mL TNF–α for 48 hours. After 24 hours at 37°C, T cells in the upper and lower chamber were recovered, and the numbers of migrating CD4⁺ and CD8⁻ TCRζbright or TCRζdim T cells were determined in each chamber by flow cytometry. Results are expressed as the percentage of cells migrating relative to the total number of each cell subset added to the transwell (TCRζbright in the lower chamber at time=24 hours/ TCRζdim at time=0 versus TCRζdim in the lower chamber time=24 hours/ TCRζdim at time=0).

Statistical Analysis
The datasets did not conform to a normal distribution. Mann–Whitney U test and Kruskall-Wallis test with Dunn multiple comparison test were used as appropriate. Wilcoxon matched paired test was used for repeated measures in the time. Spearman rank test was used to test correlations between variables. GraphPad Prism 4 and GraphPad Instat 3 softwares were used for analysis. A probability value <0.05 was considered significant.

Results

Characteristics of Patients
No significant differences in demographic and risk factors were observed between CSA group and ACS group (Table). However, differences were found in therapeutic regimens at the time of the sampling. Such differences were likely attributable to the fact that for 80% of patients in the ACS group, this hospital admission represented their first clinical manifestation of coronary disease versus only 56% in the CSA group (P=0.02). However, at 50-day follow-up there were no statistical differences regarding therapy between the two groups (P=NS; data not shown). ACS samples were obtained very early after the onset of symptoms when the elevation of troponin I were still minimal (0.4±0.0 to 5.9 ng/mL), thus ruling out the possible confounding effect of myocardial necrosis. The extent of coronary atherosclerosis was similar in patients in CSA and ACS group as documented by the number of diseased vessels in CSA, 2.0±0.8 (expressed as mean±SD), compared to ACS, 1.9±0.9 (P=0.79).

Substantial Downregulation of the TCRζ-Chain in CD4⁺ in ACS
Flow cytometric analysis (see representative dot-plots and histograms in Figure 1A through 1G) revealed a 3-fold
increased in the percentage of CD3⁺TCRζdim T cells in ACS patients (median; interquartile range: 8.2%; 3.8 to 14.5%, \( P < 0.0001 \); supplemental Table I, available online at http://atvb.ahajournals.org) compared to controls (2.6; 1.1 to 5.3%) and CSA (3.1; 1.7 to 7.0%). Confocal microscopy images confirmed the presence of CD3⁺ T cells with downregulation of the expression of TCRζ-chain in patients with ACS (Figure 1H through 1J and supplemental Figures I and II). Circulating CD4⁺ TCRζdim T cells showed a 3-fold increase in patients with ACS (5.3%; 2.6 to 9.1%; \( P < 0.0001 \), Figure 2A) when compared to controls (1.5; 0.5 to 2.9%) and CSA (1.6; 1.0 to 4.1%). The MFI index of TCRζ-chain was significantly reduced in patients with ACS in comparison to controls and patients with CSA (supplemental Figure III). To exclude that the increased levels of TCRζdim T cells reported in ACS were merely attributable to cell death, we showed that cell viability in CD3⁺ T cells were >99% out of total CD3⁺ in fresh blood, and that TCRζdim T cells were not confined to the non viable cells (supplemental Figure IV).

Differences in statin therapy are unlikely to explain the differences in TCRζ expression, as a subgroup analysis in patients without statin therapy (ACS \( n = 54 \); CSA \( n = 14 \)) confirmed a significant increase of CD4⁺ TCRζdim T cells in ACS (\( P = 0.002 \)). Moreover, statin treatment itself was unlikely to provoke such changes as by comparing CSA patients with (\( n = 18 \)) or without (\( n = 14 \)) statin therapy (respectively 1.3; 0.9 to 3.7% versus 1.8; 0.9 to 5.1%; \( P = 0.46 \)) no significant differences were found.

### Downregulation of TCRζ-Chain in CD8⁺ and NK Cells in ACS

We further assessed that downregulation of TCRζ-chain was not limited to CD4⁺ T cells in ACS. Indeed TCRζdim T cells represented 4.0% (2.0 to 7.7%) of peripheral circulating CD8⁺ T cells in patients with ACS in comparison to 0.6% (0.2 to 1.9%) in controls, and 1.2% (0.8 to 2.1%) in patients with CSA (\( P < 0.0001 \); supplemental Figure VA). Also, a 2-fold increase was observed in the levels of TCRζdim within the circulating CD3CD16⁺ NK cell subset (5.1%; 2.4 to 7.5%) in patients with ACS, whereas they accounted for 2.5% (1.3 to 3.8%) and 1.6% (0.9 to 2.7%) in controls and in patients with CSA respectively (\( P = 0.001 \); supplemental Figure VB). No significant differences were observed between control individuals and CSA patients for either T-cell or NK-cell subsets.

### Persistence of Circulating CD4⁺ TCRζdim T Cells in ACS Patients at Follow-Up

Frequencies of circulating CD4⁺ TCRζdim T cells were unchanged at a 50-day follow-up after admission (6.4; 3.7 to 10.5% versus 5.5; 3.2 to 7.7%; \( P = 0.95 \); Figure 2D), despite antiischemic therapy and risk factor management.

### Increased Frequencies of TCRζdim T Cells Are Associated With Higher CRP Levels

We observed that the frequency of CD4⁺ TCRζdim T cells, CD8⁺ TCRζdim T cells, and TCRζdim NK cells correlated with CRP levels (respectively: \( r = 0.30 \); \( P = 0.0007 \), \( r = 0.37 \); \( P =...
0.02 and \( r = 0.48; \) \( P = 0.002 \) (supplemental Figure VIA through VIC). Considering a cut-off of CRP \( \geq 2 \) mg/L used in previous studies,\(^{20} \) patients with ACS and CRP levels \( \geq 2 \) mg/L had significantly increased percentages of CD4\(^+\) TCR\(_{\text{dim}}\) T cells (7.7; 3.8 to 11.3\%, \( P = 0.003, n = 41 \)) compared to patients with ACS and low CRP levels <2 mg/L (3.3; 1.7 to 7.7\%, \( n = 25 \); Figure 3).

**Reduction of TCR\(_{\text{dim}}\)-Chain in CD4\(^+\)CD28null T Cells in Patients With ACS**

In 60 subjects, blood samples were simultaneously stained for CD3, CD4, CD28, and TCR\(_{\text{dim}}\) and analyzed by 4-color flow cytometry. We observed a significant correlation between frequencies of CD4\(^+\)CD28null T cells and CD4\(^+\) TCR\(_{\text{dim}}\) T cells (\( r = 0.43; \) \( P = 0.0006 \); Figure 4A), especially within ACS patients (\( r = 0.62; \) \( P = 0.002 \); supplemental Figure VIIA). Interestingly, a reduction of TCR\(_{\text{dim}}\)-chain expression was observed within the CD4\(^+\)CD28null subset in ACS (67.2; 39.4 to 82.9\%; \( P < 0.0001 \)), as compared to controls (20.9; 11.1 to 33.1\%; \( P < 0.001 \)) and CSA (29.2; 23.0 to 46.9\%; \( P < 0.05 \)) (Figure 4B and 4C, supplemental Figure VIIIB).

**Transendothelial Migration of TCR\(_{\text{dim}}\) T Cells, Both in CD4\(^+\) and CD8\(^+\) T Cells**

We investigated the potential role of TCR\(_{\text{dim}}\) T cells in the immunopathological pathways that drive ACS, by comparing the capacity of this subset to migrate across activated endothelium with their TCR\(_{\text{bright}}\) counterparts. An in vitro transendothelial migration assays was performed on 17 individuals enrolled in the study. Increased migration of circulating TCR\(_{\text{dim}}\) T cells (CD4\(^+\): 62.2; 48.3 to 75.9\% and CD8\(^+\): 69.0; 50.9 to 84.5\%) were observed when compared to the TCR\(_{\text{bright}}\) subset (CD4\(^+\): 47.8; 46.5 to 50.8\%; \( P = 0.005 \); and CD8\(^+\): 49.5; 42.3 to 52.4\%; \( P = \))
These observations point toward the requirement for intact controls. CD4^+ T cells migrating relative to the total number of each cell subset added to the transwell (TCR_γ^bright in the lower chamber at T=24 hours/TCR_γ^bright at T=0 versus TCR_γ^dim in the lower chamber T=24 hours/TCR_γ^dim at T=0). The proportion of migrating CD4^+ T cells was significantly higher than the proportion of migrating CD8^+ T cells (P=0.005; Figure 5 and supplemental Figure VIII). Similar migratory behavior of TCR_γ^dim T cells in comparison with their TCR_γ^bright counterparts were observed in ACS (n=5), CSA (n=6), and control (n=6) group (supplemental Figure IX). Downregulation was not attributable to the transmigration process per se, because the total number of TCR_γ^bright and TCR_γ^dim cells in top and bottom chamber does not change during the 24-hour transmigration assay.15

**Discussion**

We report for the first time expansion of circulating T cells expressing reduced levels of the invariant TCR_γ-chain in patients with ACS compared to patients with CSA and controls. CD4^+ TCR_γ^dim T cells remained elevated for at least 50 days after the acute event. In addition, we also documented that TCR_γ-chain downregulation was not limited to T cell subpopulations, but was also present in NK cells. Enrichment of TCR_γ^dim cells correlated with the magnitude of the systemic inflammatory response, raising the possibility that TCR_γ^dim cells might participate to the ongoing inflammatory and immune response in ACS.

**Functional Aspects of TCR_γ^dim T Cells in Coronary Artery Disease**

Growing similarities are emerging between atherosclerosis and chronic inflammatory diseases, including the expansion of circulating and lesional effector cells such as T-lymphocyte subsets.10 Despite T cells with low levels of TCR_γ-chain expression being refractory to TCR signaling,19 we have recently reported that TCR_γ^dim T cells express the hallmarks of memory antigen experienced effector T cells,15 produce abundant inflammatory cytokines such as TNFα and IFNγ, and promote monocyte activation through cell contact dependent pathways.15 Moreover, TCR_γ^dim T cells do not produce antiinflammatory cytokine such as IL-10,15 and do not express the T regulatory cell marker Forkhead Box Protein P3 (FoxP3; supplemental Figure X). Collectively these observations point toward the requirement for intact TCR signaling to maintain immune homeostasis through the generation or function of regulatory T cell subsets.21

Furthermore, in this study we also observed that also in patients with CAD the ability of circulating TCR_γ^dim T cells to migrate through endothelium is enhanced compared to their TCR_γ^bright counterparts. In previous study we showed also enhanced chemotactic migratory ability of TCR_γ^dim T cells in response to chemokines, such as CCL5 and CXCL10.15 Consistent with these in vitro observations, we previously documented that TCR_γ^dim T cells are enriched in synovial fluid and tissue from patients with rheumatoid arthritis.15 Moreover, in 3 patients with ACS enrolled in the study we documented a decrease in the number of CD3^+CD4^+ TCR_γ^dim T cells in blood acquired from the great cardiac vein before angioplasty compared to levels of CD3^+CD4^+ TCR_γ^dim T cells sampled from the aorta6 (supplemental Figure XI). These results could suggest an accumulation of this cell population in the coronary circulation during the acute phase of the disease. Thus, antigen experienced effector TCR_γ^dim T cells with enhanced migratory competence have the potential to extravasate and exert local proinflammatory actions in atherosclerotic plaques, thus contributing to plaque destabilization.

**Possible Mechanisms of TCR_γ-Chain Downregulation**

In our study the enrichment of circulating CD4^+ TCR_γ^dim T cell in patients with ACS was associated with higher levels of hsCRP, suggesting that the inflammatory milieu in patients with ACS may contribute to TCR_γ-chain downregulation. Antigen-dependent and antigen-independent mechanisms have been advocated to explain downregulation of the TCR_γ-chain in human and experimental models.1,14 Both mechanisms may have a role in TCR_γ-chain downregulation in ACS. For instance, prolonged exposure to bacterial and viral antigens has been shown to induce TCR_γ-chain down-regulation in vivo.14 In the setting of ACS, a variety of infectious and endogenous antigen(s) have been implicated (ie, modified low-density lipoprotein, endogenous and infectious agents-derived heat shock proteins) and could potentially lead to TCR_γ-chain downregulation.22–24 Antigen-independent mechanisms, eg, prolonged exposure inflammatory cytokines including IFNγ and TNFα, as well as reactive oxygen intermediates, have also been advocated for TCR_γ-chain downregulation.19 In our study, the downregulation of the TCR_γ-chain was not limited to CD4^+ and CD8^+ T cells but was also present in the NK-cell population, in which it may arise through FcR engagement, perhaps via immune complexes or through cytokine stimulation.14

Interestingly, the loss of CD28, as the downregulation of TCR_γ-chain, has been linked to both chronic antigen or cytokine exposure.19,25,26 CD4^+CD28null T cells, a terminally differentiated effector T cell population, was identified both in patients with rheumatoid arthritis with vascular complications and in patients with ACS.12,13,15,27 More then 65% of CD4^+CD28null T cells from patients with ACS had selectively reduced TCR_γ-chain expression. Hence, in the context of ACS, similar mechanisms might explain the reduced TCR_γ-chain expression and the loss of CD28, and the generation of T-cell subsets with perturbation of the classical immunologic synapse.
Conclusion
We show that circulating TCR_{dim}^\text{a} cells are increased in ACS, and such increase is associated with higher levels of hsCRP. TCR_{dim}^\text{a} cells display an increased ability to cross activated endothelium, and might carry the potential to accumulate in atherosclerotic plaques. Both antigen-dependent and antigen-independent mechanisms may contribute to the emergence of this unusual T-cell surface phenotype. The aberrant expression of TCR_{\zeta}-chain in circulating cell subsets further supports the role of dysregulation of the immune response in the pathogenesis of ACS. Moreover, our findings may contribute to explain the increased incidence of major cardiovascular events in systemic lupus erythematosus and rheumatoid arthritis.28

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Disclosures
None.

References
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Detailed Figure Legend 1. Flow cytometric and confocal microscopy analysis of the TCRζ-chain reveals an enrichment CD3⁺TCRζ<dim> lymphocytes and CD3⁺CD4⁺TCRζ<dim> T cells in patients with ACS compared to CSA. Triple staining was performed for CD3⁺, CD4⁺ and TCRζ-chain. Lymphocytes were gated according to forward and side-scatter (A) and CD3⁺CD4⁺ immunopositivity (B). Dot-plots are shown from one representative patient with CSA (C-D) and ACS (E-F). The percentage of CD3⁺CD4⁺TCRζ<dim>T cells out of total CD3⁺CD4⁺ T cells are indicated in the low right quadrant. (G) Representative histograms of TCRζ-chain MFI within CD3⁺CD4⁺ population from a patient with ACS (black line) and a patient with CSA (red line); and MFI index (MFI TCRζ<sub>bright</sub>/MFI TCRζ<sub>negative</sub> [shaded peak]) was shown. (H-I-J) Confocal microscopy images of a representative ACS patient. (H) TCRζ-chain (PE, red); (I) CD3 (FITC, green); (J) merging image showing examples of TCRζ<sub>bright</sub> T cell (red arrow) and TCRζ<dim> T cells (green arrows).
## Expansion of TCR\(\zeta^{\text{dim}}\)T Cells in ACS

**Ammirati et al Supplemental Data**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CSA</th>
<th>ACS</th>
<th>(P)</th>
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<tbody>
<tr>
<td><strong>T cells</strong></td>
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<td></td>
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<tr>
<td>Lymphocytes (x 10^9/L)</td>
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<td>2.0</td>
<td>2.0</td>
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<td>Interquartile</td>
<td>1.7-2.8</td>
<td>1.8-2.4</td>
<td>1.5-2.4</td>
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<tr>
<td>CD3(^+)(\zeta^{\text{dim}})/T cells (%)</td>
<td>2.6*</td>
<td>3.1†</td>
<td>8.2</td>
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<td>Interquartile</td>
<td>1.1-5.3</td>
<td>1.7-7.0</td>
<td>3.8-14.5</td>
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<tr>
<td>CD3(^+)CD4(^+) T cells (x 10^9/L)</td>
<td>1.2</td>
<td>1.3</td>
<td>1.2</td>
<td>0.64</td>
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<tr>
<td>Interquartile</td>
<td>0.8-1.5</td>
<td>1.0-1.6</td>
<td>0.9-1.6</td>
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<tr>
<td>CD3(^+)CD4(^+)(\zeta^{\text{dim}})/CD3(^+)CD4(^+) (%)</td>
<td>1.5*</td>
<td>1.7*</td>
<td>5.3</td>
<td>&lt;0.0001</td>
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<tr>
<td>Interquartile</td>
<td>0.5-2.9</td>
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<tr>
<td>CD3(^+)CD8(^+) T cells (x 10^9/L)</td>
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<td>0.7</td>
<td>0.8</td>
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<td></td>
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<tr>
<td>CD3(^+)CD8(^+)(\zeta^{\text{dim}})/CD3(^+)CD8(^+) (%)</td>
<td>0.6*</td>
<td>1.2‡</td>
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<tr>
<td>Interquartile</td>
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<td>0.8-2.1</td>
<td>2.0-5.7</td>
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<td><strong>NK cells</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CD3(^-)CD16(^+)/Lymphocytes(%)</td>
<td>6.2</td>
<td>7.0</td>
<td>6.8</td>
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<tr>
<td>Interquartile</td>
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<td>4.7-14.0</td>
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<tr>
<td>CD3(^-)CD16(^+)(\zeta^{\text{dim}})/CD3(^-)CD16(^+) (%)</td>
<td>2.5‡</td>
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<td>Interquartile</td>
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<tr>
<td><strong>CD4(^+)CD28(^\text{null}) T cells</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TCR(\zeta^{\text{dim}})/CD4(^+)CD28(^\text{null})T(%)</td>
<td>20.9*</td>
<td>29.2‡</td>
<td>67.2</td>
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<td><strong>Systemic inflammatory markers</strong></td>
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<tr>
<td>CRP (mg/L)</td>
<td>1.6*</td>
<td>2.1</td>
<td>3.2</td>
<td>0.0007</td>
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<tr>
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<td>1.0-2.5</td>
<td>1.3-3.6</td>
<td>1.6-6.0</td>
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</tbody>
</table>

* \(<0.001; †<0.01; ‡<0.05\) vs ACS after post-hoc analysis
Supplemental Table I. Phenotypic characteristics of different subsets of lymphocytes, NK cells and inflammatory profile expressed by CRP levels.
Supplemental Figure Legend

Supplemental Figure I. Representative confocal microscopy images of CD3⁺ TCRζdim T cells in an ACS patient. CD3⁺ cells were permeabilized and double stained for CD3-PB and isotype control IgG1-FITC (A), or permeabilized and double stained for CD3-PB and TCRζ-FITC (B); TCRζdim T cells are shown (green arrows). Scale bars indicated the magnification.

Supplemental Figure II. Representative confocal microscopy images in a representative control (A-B-C) and patient with ACS (D-E-F) CD3⁺ cells were sorted permeabilized and stained for DAPI, CD4⁺ (A-D) and TCRζ-FITC (B-E). Merging images were shown in C-F. In the control all T cells shown are TCRζbright, in the ACS patient 2 representative examples of TCRζdim T cells are shown (white arrows). Scale bars indicated the magnification.

Supplemental Figure III. TCRζ-chain MFI index is decreased in patients with ACS than in controls and CSA patients. Dots represent individual patient data; dashed lines show median value and continuous lines show 25th and 75th percentiles. MFI within CD3⁺CD4⁺ population (calculated as MFI TCRζbright/MFI TCRζnegative) is significant decreased in ACS patients (TCRζ-chain MFI index: 2.2) in comparison with controls (2.8; P<0.001) and patients with CSA (2.6, P<0.01). Kruskall-Wallis test with Dunn’s multiple comparison test was used.

Supplemental Figure IV. Increased levels of TCRζdim T cells in ACS are not due to cell death. Representative plots for unstained permeabilized cell (A) or an isotype control conjugated with PE (B) are shown and used to define the percentage of CD3⁺TCRζdim T cells within CD3⁺T cells (C). Cell viability was assessed using LIVE/DEAD (L/D) fixable dead cell stain kit for flow cytometry (D). Reduction of TCRζ was shown in viable T cells (E) and not confined to the dead population of T cells (F).
Supplemental Figure V. CD3⁺CD8⁺TCRζdim⁺T cells (expressed as percentage of total CD3⁺CD8⁺T, A) and CD3⁺CD16⁺TCRζdim⁺NK cells (expressed as percentage of total CD3⁺CD16⁺NK, B) were increased in patients with ACS compared with patients with CSA and controls (Kruskall-Wallis and Dunn’s test). Dashed lines show median, continuous lines show 25th and 75th percentiles.

Supplemental Figure VI. Expansion of circulating TCRζdim⁺ cells is associated with higher levels of hsCRP. Correlation between levels of CRP (mg/l) and (A) CD3⁺CD4⁺TCRζdim⁺T cells, (B) CD3⁻CD16⁺TCRζdim⁺NK cells and (C) CD3⁺CD8⁺TCRζdim⁺T cells (Spearman’s rank test).

Supplemental Figure VII. TCRζdim⁺ and CD28null⁺T cells represent overlapping but distinct T cell subsets. (A) Correlation between CD4⁺TCRζdim⁺T cells and CD4⁺CD28null⁺ T cells (expressed as percentage of total CD3⁺CD4⁺ T cells) in patients with ACS (Spearman’s rank test). (B) Representative dot plots from a control and from a patient with CSA; showing CD4⁺TCRζdim⁺ T cell, CD4⁺CD28null⁺ T-cell subsets and the percentage of TCRζdim⁺ cells within CD4⁺CD28null⁺ T cells (percentage of these subsets are reported in the low left quadrants). It is possible to see how, within CD4⁺CD28null⁺ T cells the percentage of TCRζdim⁺ is decreased in patient with CSA and healthy controls in comparison to patient with ACS (See Figure 4C). Results are summarized in Figure 4B.

Supplemental Figure VIII. TCRζdim⁺ T cells exhibit enhanced migratory capacity in vitro. PBL (input, at time [T]=0) were applied on transwell containing a monolayer of TNFα-stimulated HUVEC and cells in upper and lower chambers harvested at 24 hours (T=24hr) prior to staining for expression of CD4 (n=17) and CD8 (n=17) and TCRζ–chain and analysis by flow cytometry. A representative flow cytometry analysis is shown: in A it is reported lymphocyte distribution at T=0 and at T=24hr, in B it is reported CD4⁺ T-cell distribution T=0 and at T=24hr.
**Supplemental Figure IX.** TCR$_{\zeta}^{dim}$ T cells exhibit similar enhanced migratory capacity in vitro in controls, and patients with CSA and ACS. PBL (input, at time [T]=0) were applied on transwell containing a monolayer of TNF$\alpha$-stimulated HUVEC and cells in upper and lower chambers harvested at 24 hours (T=24hr) prior to staining for expression of CD4 (n=17: 5 controls, 6 CSA, 6 ACS) and CD8 (n=17: 5 controls, 6 CSA, 6 ACS) and TCR$\zeta$-chain and analysis by flow cytometry. Results comparing TCR$_{\zeta}^{bright}$ with TCR$_{\zeta}^{dim}$ migratory ability are expressed as the percentage of cells migrating relative to the total number of each cell subset added to the transwell (TCR$_{\zeta}^{bright}$ in the lower chamber at T=24hr / TCR$_{\zeta}^{bright}$ at T=0 vs TCR$_{\zeta}^{dim}$ in the lower chamber T=24hr / TCR$_{\zeta}^{dim}$ at T=0, See representative experiment in Figure 5A). Kruskall-Wallis test with Dunn’s multiple comparison test was used.

**Supplemental Figure X.** Flow cytometry characterization showed that CD4$^+$ TCR$_{\zeta}^{dim}$ T cells do not express FoxP3. CD3$^+$CD4$^+$ T cells (A) were permeabilized, (B) and stained for TCR$\zeta$-chain and FoxP3 (C). Nearly all FoxP3$^+$ T cells express TCR$\zeta$-chain (upper right quadrant, C). Representative experiment of 3 experiments performed.

**Supplemental Figure XI.** Reduction of TCR$_{\zeta}^{dim}$ T cells across coronary thrombotic lesion during myocardial infarction. Triple staining was performed for CD3$^+$, CD4$^+$ and TCR$\zeta$. Results shown are from one representative patient. A decrease in the number of CD3$^+$CD4$^+$ TCR$_{\zeta}^{dim}$ T cells in blood acquired from the great cardiac vein before angioplasty compared to levels of CD3$^+$CD4$^+$ TCR$_{\zeta}^{dim}$ T cells sampled from the aorta. The percentage of CD3$^+$ TCR$_{\zeta}^{dim}$ (A) and CD3$^+$CD4$^+$ TCR$_{\zeta}^{dim}$ (B) T cells are indicated in the lower right quadrant.
Supplemental Figure I
Supplemental Figure III

TCRζ MFI in CD3+CD4+ T-cells

- Controls (n=42)
- CSA (n=32)
- ACS (n=66)

P<0.001
P<0.01
Supplemental Figure IV
Supplemental Figure V
Supplemental Figure VII

B) Control

A) 
\[ \frac{CD^4+CD28null}{CD^4+} (\%) \] vs. \[ CD^4+ TCR\zeta^{dim}/ CD^4+ (\%) \]

\[ P=0.002 \]
\[ r=0.62 \]

P=0.002
r=0.62

CD4+CD28null/CD4+(%)
Supplemental Figure VIII
Supplemental Figure IX

A) % CD4+ cell migrating

P=0.57

Controls (n=5)  CSA (n=6)  ACS (n=6)

B) % CD8+ cell migrating

P=0.81

Controls (n=5)  CSA (n=6)  ACS (n=6)
Supplemental Figure X

A) Sorted Cells

B) Unstained Permeabilized

C) TCRz FITC, FoxP3 stained

- CD3+CD4+ TCRz FITC, FoxP3 stained
- FoxP3 PB

- Unstained Permeabilized

- TCRz FITC, FoxP3 stained

- FoxP3 PB

- Sorted Cells

- FSC-A vs SSC-A
Supplemental Figure XI