T lymphocytes and fractalkine contribute to myocardial ischemia/reperfusion injury in patients

Stephen E. Boag, …, Bernard Keavney, Ioakim Spyridopoulos


**BACKGROUND.** Lymphocytes contribute to ischemia/reperfusion (I/R) injury in several organ systems, but their relevance in ST elevation myocardial infarction (STEMI) is unknown. Our goal was to characterize lymphocyte dynamics in individuals after primary percutaneous coronary intervention (PPCI), assess the prognostic relevance of these cells, and explore mechanisms of lymphocyte-associated injury.

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CONCLUSIONS. Lymphopenia following PPCI is associated with poor prognosis. Our data suggest that fractalkine contributes to lymphocyte shifts, which may influence development of MVO through the action of effector T cells.

TRIAL REGISTRATION. Not applicable.

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Introduction

Primary percutaneous coronary intervention (PPCI) is now standard of care for the treatment of acute ST elevation myocardial infarction (STEMI). Although PPCI results in reduction in both mortality and morbidity compared with thrombolytic therapy, ischemia/reperfusion (I/R) injury remains an important complication, contributing up to 50% of final infarct size (1). I/R injury is a multifactorial process, to which metabolic factors (2), inflammation (3), and microvascular obstruction (MVO) (4) contribute. To date, no treatments targeting this process have shown conclusive benefit.

While the role of T lymphocytes (T cells) in I/R injury relating to other organ systems has been well studied (5), relatively little evidence exists concerning myocardial I/R injury. Two studies by Yang et al. have suggested a critical role for IFN-γ producing CD4+ T cells in a mouse model of myocardial I/R injury (6, 7). However, the significance of these cells in human patients undergoing PPCI for STEMI has yet to be studied.

While their specific contribution is not currently understood, indirect evidence points to a role for lymphocytes in myocardial infarction (MI) and I/R injury in humans. Several studies have identified poor prognostic significance of lymphopenia (8) or high neutrophil/lymphocyte ratio (9–13), although the type of infarct and treatment strategy have been variable. Furthermore, one study has identified a correlation between lymphopenia and MVO following PPCI (14). The goal of our study was to assess the prognostic impact of transient lymphopenia in a well-defined population undergoing PPCI for STEMI, clarify the subpopulations of cells involved, and investigate possible mechanisms.

Results

Lymphopenia after PPCI predicts long-term mortality. First, we wanted to determine whether lymphopenia predicted outcome in a well-defined population of STEMI patients undergoing PPCI. We retrospectively analyzed the lymphocyte counts of 1,377 consecu-
Table 1. Baseline data for 1,377 patients included in retrospective analysis of mortality data

<table>
<thead>
<tr>
<th>Minimum lymphocyte tertile</th>
<th>Low (&lt;1,300 cells/μl)</th>
<th>Middle (1,300–1,910 cells/μl)</th>
<th>High (&gt;1,910/μl)</th>
<th>n</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>68.1 ± 13.5</td>
<td>62.1 ± 12.9</td>
<td>58.3 ± 11.7</td>
<td>1,377</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>327 (71.2)</td>
<td>329 (71.3)</td>
<td>336 (73.2)</td>
<td>1,377</td>
<td>0.785</td>
</tr>
<tr>
<td>BMI</td>
<td>26.6 ± 5.1</td>
<td>27.2 ± 5.2</td>
<td>28.3 ± 5.4</td>
<td>1,222</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>51 (11.2)</td>
<td>42 (9.2)</td>
<td>51 (11.1)</td>
<td>1,377</td>
<td>0.526</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>168 (42.7)</td>
<td>183 (43.4)</td>
<td>202 (47.5)</td>
<td>1,240</td>
<td>0.32</td>
</tr>
<tr>
<td>Hypertension</td>
<td>206 (44.9)</td>
<td>184 (40.0)</td>
<td>163 (35.3)</td>
<td>1,377</td>
<td>0.015</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>131 (28.5)</td>
<td>137 (29.8)</td>
<td>138 (30.1)</td>
<td>1,377</td>
<td>0.861</td>
</tr>
<tr>
<td>Previous MI</td>
<td>82 (18.4)</td>
<td>66 (14.8)</td>
<td>46 (10.1)</td>
<td>1,368</td>
<td>0.003</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>39 (8.5)</td>
<td>34 (7.4)</td>
<td>20 (4.4)</td>
<td>1,374</td>
<td>0.034</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>82 (18.4)</td>
<td>66 (14.8)</td>
<td>46 (10.1)</td>
<td>1,374</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>39 (8.5)</td>
<td>34 (7.4)</td>
<td>20 (4.4)</td>
<td>1,374</td>
<td>0.008</td>
</tr>
<tr>
<td>PVD</td>
<td>336 (73.2)</td>
<td>336 (73.2)</td>
<td>336 (73.2)</td>
<td>1,377</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Door to balloon time (min)</td>
<td>30.9 ± 18.5</td>
<td>29.3 ± 18.5</td>
<td>28.5 ± 17.5</td>
<td>1348</td>
<td>0.152</td>
</tr>
<tr>
<td>Total ischemic time (min)</td>
<td>276.4 ± 366.8</td>
<td>247.4 ± 238.2</td>
<td>283.6 ± 487.1</td>
<td>1329</td>
<td>0.320</td>
</tr>
<tr>
<td>Cardiogenic shock</td>
<td>22 (4.8)</td>
<td>8 (1.7)</td>
<td>10 (2.2)</td>
<td>1,367</td>
<td>0.012</td>
</tr>
<tr>
<td>Anterior MI</td>
<td>205 (44.9)</td>
<td>174 (38.3)</td>
<td>155 (33.9)</td>
<td>1,368</td>
<td>0.003</td>
</tr>
<tr>
<td>Multivessel PCI</td>
<td>65 (14.2)</td>
<td>41 (9.8)</td>
<td>40 (8.7)</td>
<td>1,377</td>
<td>0.01</td>
</tr>
<tr>
<td>TIMI 3 post-PCI</td>
<td>382 (89)</td>
<td>401 (93)</td>
<td>420 (95.2)</td>
<td>1,301</td>
<td>0.002</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>135.8 ± 17.1</td>
<td>139.7 ± 16.8</td>
<td>143.0 ± 14.9</td>
<td>1374</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>4.7 ± 1.3</td>
<td>5.1 ± 1.3</td>
<td>5.3 ± 1.3</td>
<td>1283</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>113.3 ± 69.0</td>
<td>98.8 ± 24.1</td>
<td>96.1 ± 22.2</td>
<td>1373</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin</td>
<td>399 (86.9)</td>
<td>416 (90.6)</td>
<td>423 (92.2)</td>
<td>1377</td>
<td>0.026</td>
</tr>
<tr>
<td>ACE inhibitor/ARB</td>
<td>374 (81.5)</td>
<td>407 (88.7)</td>
<td>410 (89.3)</td>
<td>1377</td>
<td>0.001</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>366 (79.7)</td>
<td>392 (85.4)</td>
<td>400 (87.1)</td>
<td>1377</td>
<td>0.006</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>404 (88.0)</td>
<td>420 (91.5)</td>
<td>428 (93.2)</td>
<td>1377</td>
<td>0.019</td>
</tr>
<tr>
<td>Aspirin</td>
<td>406 (88.5)</td>
<td>421 (91.7)</td>
<td>429 (93.5)</td>
<td>1377</td>
<td>0.025</td>
</tr>
<tr>
<td>Glycoprotein IIb/IIIa inhibitor</td>
<td>351 (76.8)</td>
<td>395 (86.6)</td>
<td>405 (88.4)</td>
<td>1371</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Continuous variables expressed as mean ± SD and compared using one-way ANOVA; categorical variables as n (%) and compared using χ² test. CAD, coronary artery disease; CVA, cerebrovascular accident; TIA, transient ischemic attack; PVD, peripheral vascular disease; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker. “Diabetes mellitus” refers to types 1 and 2.
not seen in the NSTEMI group (before PCI: 1,899 ± 207 cells/μl, 90 minutes: 1,689 ± 137 cells/μl, P = 0.10). Thus, lymphocyte counts were significantly lower in the STEMI compared with the NSTEMI at 90 minutes (P = 0.012), although the level was nonsignificantly elevated before PCI. The temporal evolution of other leukocyte subsets in the STEMI group also showed distinct dynamics. There was a small drop in monocyte counts by 90 minutes (776 ± 36 cells/μl, P = 0.039), followed by a peak at 24 hours (898 ± 58 cells/μl at 90 minutes (P < 0.001, Figure 3D), prior to recovery by 24 hours. In contrast, a significant decline was not seen in the NSTEMI group (1,275 ± 159 cells/μl at 1,234 ± 118 cells/μl, P = 0.11). Similarly, NK cells showed a large drop in the STEMI group, from 501 ± 33 cells/μl to 203 ± 18 cells/μl at 90 minutes (P < 0.001, Figure 3G), although they did not significantly recover by 24 hours. Unlike in T cells, however, the drop in NK cells in the STEMI group was paralleled by a similar, albeit slightly smaller, drop in the NSTEMI group (397 ± 65 to 216 ± 24 cells/μl at 90 minutes, P = 0.004).

Highly differentiated effector T cell subsets show greater decline following reperfusion in STEMI. Having established the transient loss of circulating T cells following reperfusion in STEMI, we focused on the behavior of the subpopulations (Figure 3, E and F, and Figure 4). These were assessed using the percentage change in cell counts, with the total drop best demonstrated by the change occurring between pre-reperfusion and 90 minutes (Figure 4, C and D). While total CD4+ T cell levels dropped by a median of ~29% (interquartile range [IQR] ~43%, ~15%), CD8+ T cells dropped by ~55% (IQR ~66%, ~29%). Comparable drops were not observed in the NSTEMI population (median change of +8% and ~3% for CD4+ and CD8+ T cells, respectively; P < 0.001, STEMI vs. NSTEMI for each subset). Furthermore, within these two major T cell subsets, depletion varied between the subpopulations, with a progressively greater drop in the more differentiated cells (Figure 4, C and D). Thus, the effector cells declined by ~52% (~67%, ~57%) in the case of CD8+ effector memory (TEMRA) and by ~66% (~74%, ~44%) in terminally differentiated CD8+CD45RA+ effector memory (TEM) cells, compared with only ~26% (~40%, ~5%) for CD8+ naive (TNaive) cells and ~26% (~43%, ~6%) for CD8+ central memory (TCM) cells (Figure 4D). A comparable trend was also seen within the equivalent CD4+ T cell subsets (Figure 4C). Moreover, when the effector T cell subsets were further divided by expression of the co-stimulatory molecule CD27, which is downregulated in highly differentiated T cells, the drop was greater still in cells lacking expression (Supplemental Figure 1). The same pattern in cell level drops was seen in the early post-reperfusion interval between 15 and 30 minutes (Figure 4, A and B). In general, the
Table 2. Baseline data for STEMI and NSTEMI patients included in prospective lymphocyte subpopulation study

<table>
<thead>
<tr>
<th></th>
<th>STEMI (n = 59)</th>
<th>NSTEMI (n = 15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.3 ± 10.7</td>
<td>61.1 ± 11.8</td>
<td>0.742</td>
</tr>
<tr>
<td>Male</td>
<td>44 (74.6)</td>
<td>11 (73.3)</td>
<td>1.000</td>
</tr>
<tr>
<td>BMI</td>
<td>26.8 ± 4.6</td>
<td>30.9 ± 6.0</td>
<td>0.006</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (10.2)</td>
<td>0 (0)</td>
<td>0.337</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>23 (39.0)</td>
<td>7 (46.7)</td>
<td>0.697</td>
</tr>
<tr>
<td>Active smoker</td>
<td>31 (52.5)</td>
<td>4 (26.7)</td>
<td>0.089</td>
</tr>
<tr>
<td>Hypertension</td>
<td>19 (32.2)</td>
<td>10 (66.7)</td>
<td>0.020</td>
</tr>
<tr>
<td>Anterior MI</td>
<td>28 (47.5)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>5.3 ± 1.1</td>
<td>4.3 ± 1.1</td>
<td>0.036</td>
</tr>
<tr>
<td>Peak troponin T (ng/l)</td>
<td>4899 ± 3385</td>
<td>207 ± 144.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Procedural characteristics

- Door-to-balloon time (minutes) | 26.8 ± 14.3 | N/A | N/A
- Onset-to-refluxion time (minutes) | 164.6 ± 81.3 | N/A | N/A
- Onset-to-procedure time (days) | N/A | 5.0 ± 2.7 | 0.001
- Pre PCI flow (TIMI 0/1/2/3) | 55/4/0/0 | 2/1/0/12 | <0.001
- Post PCI flow (TIMI 0/1/2/3) | 0/0/0/59 | 1/0/0/14 | 0.203
- Vascular access (radial/femoral) | 56/3 | 13/2 | 0.265

Pre-hospitalization medication

- Statin therapy | 10 (16.9) | 6 (40.0) | 0.077
- β-blocker | 2 (3.4) | 3 (20.0) | 0.054
- Aspirin | 5 (8.5) | 2 (13.3) | 0.624
- ACE inhibitor/ARB | 6 (10.2) | 7 (46.7) | 0.003

Continuous variables expressed as mean ± SD and compared using Mann-Whitney U test; categorical variables as n (%) and compared using χ² or Fisher’s exact test as appropriate.

Table 2. Baseline data for STEMI and NSTEMI patients included in prospective lymphocyte subpopulation study

Counts were small, they were well above the coefficient of variation (CV) of the measurement (1.5%, 1.6%, and 2% for total T cells, CD4+ cells, and CD8+ T cells, respectively; Supplemental Table 4), indicating that they were within the range detectable with this assay. In contrast, in anterior MI cases where the samples were taken later than 45 minutes, and in inferior MI (where the affected myocardium is primarily not drained via the CS), no significant transcoronary gradient was seen (Figure 5B).

Transient depletion of highly differentiated T cell subsets is associated with MVO. Next we investigated whether the changes observed in cell counts were associated with a marker of myocardial I/R injury on cardiac MRI. MVO is known to be one component of I/R injury and can be readily detected on late gadolinium enhancement (LGE) MRI images as an area of hypoenhancement within the infarct core (Figure 6A). To study this phenomenon, we divided STEMI patients into three tertiles based on the presence and extent of MVO (zero, low, and high MVO; see Supplemental Tables 5 and 6 for group characteristics) and compared the dynamic changes in cell counts between each of these tertiles. It was found that the early changes, occurring between 15 and 30 minutes after reperfusion, showed a relationship with the development of MVO (Supplemental Table 7 and Figure 6, C and D). In particular, the early drop in T cell counts demonstrated a very strong association with MVO tertile (P = 0.003), with a greater drop in patients with high MVO. In comparison, there was only a weak relationship for monocytes (P = 0.049) and none for granulocytes, B cells, or NK cells (Supplemental Table 7). The high-MVO group displayed a significantly greater drop than the zero-MVO group for both CD4+ (-14.3% ± 1.8% vs. -6.0% ± 1.4%, P = 0.012) and CD8+ T cells (-27.1% ± 3.6% vs. -13.9% ± 2.5%, P = 0.020) (Figure 6, C and D). Furthermore, when the drops in the effector T cell subsets (TEm and TEMRA cells) were analyzed separately, the relationship with MVO was more striking still. In the case of CD4+ TEm cells, the high-MVO group had a mean drop of -21.1% ± 2.3% compared with -8.6% ± 2.1% in the zero-MVO group (P = 0.005). The strongest relationship of any cell population, however, was seen in the scarce CD4+ TEMRA cells (high-MVO mean drop: -27.8% ± 3.2% vs. zero-MVO: -7.8% ± 2.9%, P < 0.001). The analysis was also carried out with exclusion of cases with trivial non-physiological (thrombolysis in myocardial infarction [TIMI] grade 1) arterial flow prior to reperfusion in order to assess whether inclusion of these cases affected the results. While significance values were slightly lower due to the smaller case number, the overall findings were unaltered (Supplemental Table 8).

Effectors of the immune system are involved in the healing of heart tissue after MI. They help to prevent the formation of scar tissue and initiate cardiac repair. This process is mediated by T cell subsets, which have different functions and characteristics. For example, CD4+ TEm cells are known to possess potent effector functions, such as cytokine production, in contrast to the effector T cell subsets (TEM and TEMRA) which are less differentiated and have a lower proliferative capacity. Such differences are driven by microenvironmental and extracellular matrix factors.

In order to assess the potential role of T cell subsets in myocardial repair, we conducted a lymphocyte subpopulation study. We included transcoronary samples from patients with ST-elevation myocardial infarction (STEMI) and non-ST-elevation myocardial infarction (NSTEMI) and studied the change in lymphocyte subpopulations following reperfusion. We found that the early changes in lymphocyte subsets occurred between 15 and 30 minutes after reperfusion, and these changes were associated with the development of myocardial microvascular obstruction (MVO). The high-MVO group showed a significantly greater drop in the effector T cell subsets (TEm and TEMRA cells) compared to the zero-MVO group. Furthermore, when the drops in the TEMRA cells were analyzed separately, the relationship with MVO was more pronounced. These findings suggest that T cell subsets play a role in the healing process after MI and that the early changes in lymphocyte subsets are associated with the development of MVO.
as showing a strong relationship ($r^2 = 0.99$, $P = 0.006$) between expression on T cell subsets and the cellular drop (Supplemental Table 9 and Figure 7C). Moreover, when we analyzed gene transcription for a variety of chemokine and adhesion receptors in peripheral blood mononuclear cells (PBMCs) from STEMI patients undergoing PPCI, the same receptor stood out as showing the most dramatic and consistent change: compared with expression levels 3 months after reperfusion, CX3CR1 was initially downregulated prior to reperfusion, then upregulated 24 hours afterward (Supplemental Table 10). CX3CR1 is the receptor for fractalkine (CX3CL1), a unique chemokine known to play a key role in migration and adhesion of effector T cells and NK cells to vascular endothelium (18, 19). Indeed, expression of CX3CR1 varied significantly between T cell subsets and was higher in CCR7– effector cells (Figure 7D and E). Consequently, we then investigated the dynamics of the CX3CR1 ligand soluble fractalkine (sFKN) in the serum of STEMI patients. Despite an initial drop in the first 15 minutes after reperfusion, sFKN reached a peak concentration at 90 minutes, coinciding with the observed nadir in T cell counts (Figure 7F).

Last, we wanted to further assess the interaction between sFKN and CX3CR1 in T cells following reperfusion. We analyzed CX3CR1 expression over time and found a clear initial decrease in CX3CR1 expression in effector CD4+ and CD8+ T cells, prior to partial recovery by 24 hours (Figure 7G). This apparent decrease in CX3CR1 expression could be due to one of three possibilities: cell-by-cell downregulation, selective loss from the bloodstream of CX3CR1-expressing cells, or an interaction with the circulating ligand (sFKN). It was noteworthy that this finding was in contrast to the observed upregulation of CX3CR1 in PBMCs from the gene transcription data (Supplemental Table 10). We speculated that this discrepancy could be related to a ligand-receptor interaction and therefore performed an in vitro competition assay to assess the impact of fractalkine binding on measured CX3CR1 expression. This showed that in CX3CR1-expressing cells, the addition of fractalkine leads to a reduction in CX3CR1 MFI, providing in vitro evidence of ligand-receptor binding and possibly explaining the above discrepancy (Figure 7H).

Discussion

In this study we have shown that lymphopenia during admission is predictive of poor long-term outcome in STEMI treated with PPCI. We have clarified the temporal evolution of lymphocyte dynamics in the blood following reperfusion and shown that lymphopenia in this setting is primarily due to depletion of effector T cell subsets and NK cells. The loss of T cells from the circulation is transient, reaching nadir at 90 minutes, with recovery almost
complete by 24 hours. Furthermore, we have identified an association between the development of MVO, a component of I/R injury, and the extent of early depletion of effector T cell subsets. This provides what we believe to be the first evidence in humans of a role for these cells in myocardial I/R injury, adding to previously published data from mouse models (6, 7). We have then gone on to elucidate the mechanism of lymphocyte depletion, identifying the chemokine fractalkine as the prime candidate for a central role in this process.

Lymphopenia predicts poor outcome in STEMI treated with PPCI. Until now, the importance of lymphocyte counts in STEMI patients undergoing PPCI has not been fully elucidated. Lymphopenia has previously been shown in a study of 1,037 patients by Dragu et al. to be an independent predictor of long-term mortality in acute myocardial infarction (8). However, this study included both STEMI and NSTEMI patients, managed with a variety of treatment modalities. Furthermore, numerous studies have assessed the predictive value of neutrophil-to-lymphocyte ratio in STEMI treated with PPCI, finding it to be an independent predictor of outcome, in terms of both mortality (13) and major adverse cardiovascular events (10, 12). Our study, however, is the first to our knowledge to directly assess the prognostic significance of lymphocyte counts alone following PPCI for STEMI. We have shown that patients with a lymphocyte count in the lowest tertile have significantly greater mortality over 40 months. Furthermore, while most previous studies have focused on blood tests taken on presentation, we have specifically studied the minimum lymphocyte count observed during the admission. This is of particular relevance, given the significant temporal evolution in lymphocyte counts following reperfusion.

Transient T cell depletion from the bloodstream is due to selective loss of effector T cell subsets and occurs secondary to I/R. Our data provide clear evidence of lymphocyte depletion from circulating blood following reperfusion, with a detailed analysis of the specific subsets involved. T cell depletion is primarily due to an acute decrease in both CD4+ and CD8+ effector T cells (TEM and TEMRA). In contrast to less differentiated T cell subsets (T_N and T_CM), these cells are

Figure 3. Time courses in circulating leukocyte subset counts. (A–C) Major leukocyte subsets of (A) lymphocytes, (B) monocytes, and (C) granulocytes, showing change in cell counts over time. Time points were measured from reperfusion in the STEMI group and from first culprit vessel instrumentation (or initial “Pre” blood sampling if no intervention occurred in the NSTEMI group). (D–F) T lymphocyte cell counts, including (D) total T cells, (E) CD4+ T cells, and (F) CD8+ T cells. (G and H) CD3– (non-T) lymphocyte subset counts: (G) NK cells and (H) B cells. Upper statistics (red) refer to differences in counts between the indicated time points in the STEMI group (Friedman test, with Dunn’s multiple-comparisons test); while lower statistics (black) refer to difference between STEMI and NSTEMI at corresponding time points (Mann-Whitney U test) (STEMI n = 59, NSTEMI n = 15). *P < 0.05, **P < 0.01, ***P < 0.001.
characterized by migration to inflamed tissues and potent effector functions, including production of proinflammatory cytokines such as IFN-γ and TNF-α (15). Importantly, this characteristic loss of effector T cells was seen only in STEMI patients and not in the NSTEMI control group. The NSTEMI and STEMI patients do undoubtedly represent different clinical scenarios, with significantly larger infarcts in the STEMI group and primarily open infarct–related arteries at presentation in the NSTEMI group. Given this latter characteristic, the NSTEMI patients will not have experienced acute I/R. They did, however, undergo the same procedure, and consequently, we believe that this indicates that the characteristic cellular changes seen in the STEMI group were not merely procedurally induced, but were secondary to the I/R process. There are some additional limitations to the use of the NSTEMI group as a control in this setting, most notably the markedly different infarct size, as indicated by the peak troponin T level (Table 2). However, the only potential experiment that could avoid this problem would be to compare STEMI patients undergoing reperfusion with similar cases without reperfusion, which would clearly not be ethically acceptable in a human population.

Our transcoronary gradient data suggest that in STEMI patients following reperfusion, some T cells are lost within the myocardium. Blood was taken simultaneously from the aortic root and CS at the end of PPCI in a subset of STEMI patients (n = 12). Of these, n = 9 were anterior STEMI (samples taken at <45 minutes after reperfusion in n = 6; >45 minutes after reperfusion in n = 3) and n = 3 were inferior STEMI (all sampled at <45 minutes). (A) Percentage change in cell counts of leukocyte subsets between aorta (Ao) and CS for anterior MI with sampling at <45 minutes (n = 6). Negative values indicate a drop across myocardial circulation. Statistics refer to Wilcoxon signed rank test of aorta versus CS counts for the indicated populations. (B) Impact of sample timing and infarct location on transcoronary gradients. Statistics refer to difference between anterior infarcts with sampling before (n = 6) or after (n = 3) 45 minutes (Mann-Whitney U test). Note: Mean troponin T for anterior infarcts with sampling <45 minutes: 4,814 ± 1,811 ng/l, anterior infarcts with sampling >45 minutes: 9,705 ± 2,409 ng/l, inferior infarcts: 5,462 ± 2,890 ng/l, excluding larger infarcts in the anterior sampling <45 minutes cases as a possible cause for these findings. *P < 0.05.
Effector T cells from the circulating blood early after reperfusion is strongly associated with development of MVO, one component of I/R injury, in contrast to primarily negative findings in other leukocyte populations. This further elucidates the work of Bodi et al, who have previously shown an association between lymphopenia after PPCI and MVO (14). The process of MVO is multifactorial, involving direct endothelial damage, leukocyte plugging of the microvasculature, platelet/fibrin embolization, and myocyte swelling (4). Two previous studies have shown an association between neutrophil/lymphocyte ratio (20) and platelet/lymphocyte ratio (21), respectively, and poor myocardial perfusion following PPCI. While our findings demonstrate an association between effector T cell depletion and MVO, they are not conclusive regarding a direct causative relationship. However, taken in the context of previously published research in mice showing a role for IFN-γ-producing CD4+ T cells in myocardial I/R injury (6, 7), we believe this to be the first evidence in humans of such a potential link. We propose that effector T cells may contribute to MVO through direct trapping within the myocardial microvasculature, where they release inflammatory mediators, contributing to further leukocyte infiltration and myocardial damage.

At present, no specific treatment targeting myocardial I/R injury has reached routine clinical use, and most interventions have proved disappointing (22). However, one small proof-of-concept study has shown a potential benefit with administration of cyclosporine immediately prior to PPCI (23). While the rationale for this treatment was prevention of mitochondrial permeability transition pore opening, a key event in I/R injury, it is also noteworthy that it has a profound effect on T cells, limiting their activation through the inhibition of calcineurin (24). It is therefore possible that any beneficial effect in myocardial I/R injury could be mediated at least in part through this mechanism.

Effector T cells are likely to contribute to myocardial I/R injury following PPCI for STEMI. We have shown that greater loss of effector T cells from the circulating blood early after reperfusion is strongly associated with development of MVO, one component of I/R injury, in contrast to primarily negative findings in other leukocyte populations. This further elucidates the work of Bodi et al, who have previously shown an association between lymphopenia after PPCI and MVO (14). The process of MVO is multifactorial, involving direct endothelial damage, leukocyte plugging of the microvasculature, platelet/fibrin embolization, and myocyte swelling (4). Two previous studies have shown an association between neutrophil/lymphocyte ratio (20) and platelet/lymphocyte ratio (21), respectively, and poor myocardial perfusion following PPCI. While our findings demonstrate an association between effector T cell depletion and MVO, they are not conclusive regarding a direct causative relationship. However, taken in the context of previously published research in mice showing a role for IFN-γ-producing CD4+ T cells in myocardial I/R injury (6, 7), we believe this to be the first evidence in humans of such a potential link. We propose that effector T cells may contribute to MVO through direct trapping within the myocardial microvasculature, where they release inflammatory mediators, contributing to further leukocyte infiltration and myocardial damage.

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**Serum fractalkine dynamics and T cell subset CX3CR1 expression suggest a critical role for this chemokine.** Fractalkine is a unique chemokine that exists as a membrane-bound adhesion molecule, where it contributes to leukocyte binding and arrest on the vas-
chemokine receptors expressed on lymphocytes as showing a striking correlation between expression in T cell subsets and their depletion from the circulation following reperfusion in STEMI. In keeping with one other study investigating fractalkine levels in this setting (28), we have also shown a peak in sFKN, as well as an increase in gene transcription for CX3CR1, following reperfusion. Prior to this rise in sFKN, however, there was an early drop by 15 minutes after reperfusion. We subsequently demonstrated in vitro evidence of binding between CX3CR1 on the surface of lymphocytes and recombinant soluble fractalkine. We hypothesize...
that following reperfusion, any available sFKN is rapidly bound by CX3CR1-expressing leukocytes, many of which also bind membrane-bound fractalkine on inflamed vascular endothelium, resulting in their margination from the circulation. Continued release of sFKN from activated, inflamed endothelium may be augmented secondary to inflammatory cytokine production by effector T cells, leading to the peak seen at 90 minutes, coinciding with the nadir in circulating T cell counts. Furthermore, given the role of fractalkine in leukocyte margination, and the contribution of leukocyte plugging to the development of MVO, it is conceivable that fractalkine-mediated binding of leukocytes to vascular endothelium within the reperfused microcirculation could affect the extent of MVO. This chemokine is known to mediate effector T cell sequestration in a number of other disease processes, including atopic dermatitis (29), rheumatoid arthritis (30), and multiple sclerosis (31), in each of which these cells are...
thought to contribute critically to disease mechanisms. Moreover, in an in vitro experimental model using leukocytes from human CMV-positive individuals, stimulated PBMCs have been shown to cause endothelial cell damage in a CX3CR1/fractalkine-dependent manner (27). Leukocyte plugging and endothelial damage are known to be major contributing causes of MVO (4, 32) and represent plausible mechanisms through which T cells could mediate this effect. Release of inflammatory cytokines by effector T cells may also induce further endothelial activation and infiltration of other leukocytes, including neutrophils, compounding the problem. This hypothesis is supported by previously published murine data demonstrating that T cell deficiency in Rag1-knockout mice reduces myocardial neutrophil infiltration and protects against I/R injury, while reconstitution with CD4+ T cells able to produce IFN-γ abolishes this protection (7). It is noteworthy, however, that in our study even T cell subsets not expressing CX3CR1 (e.g., CCR7+CD4+ T cells and B cells) displayed an approximately 20% drop in circulating counts after reperfusion, indicating that signaling through this chemokine receptor is not the sole mechanism behind lymphocyte depletion.

**Study limitations.** The principal limitations of this study relate to the difficulties in demonstrating causation in disease processes in human subjects. Thus, our findings regarding the relationship between effector T cell dynamics and MVO are correlative in nature. The same limitation affects our findings of a correlation between effector T cell dynamics and MI length. The difficulty of achieving a model that closely mirrors the clinical setting of STEMI treated by PPCI. This allows us to advance our understanding and develop new hypotheses, which can perhaps go on to be tested in animal models. While the involvement of the acquired immune system in myocardial I/R injury, it must be recognized that we are unable to categorically prove causation through analysis of human patients’ samples. To do so would require blockade and reconstitution of an effect in an animal model, for instance through deletion of CX3CR1 or fractalkine in a murine myocardial I/R model. However, such strategies also have substantial disadvantages, most notably the difficulty of achieving a model that suitably replicates the clinical scenario in humans, as well as the significant differences that exist between the human and murine immune systems. Consequently, our study has the advantage of directly investigating the human immune system in the real clinical setting of STEMI treated by PPCI. This allows us to advance our understanding and develop new hypotheses, which can perhaps go on to be tested in animal models. While the involvement of the acquired immune system in myocardial injury and repair is gaining increasing interest (33), it remains significantly underinvestigated, and further studies would be greatly welcomed.

One further possible limitation in our study, however, could be the relatively homogenous total ischemic times of the STEMI population. In order to investigate I/R injury, it was necessary to ensure that the total ischemic time was not overly long, with the infarct having already been completed prior to reperfusion. Consequently, the inclusion criteria for our prospective cohort was limited to STEMI patients with total ischemic times of less than 6 hours. Although this was important to ensure an appropriate study sample, it must be borne in mind that in the clinical setting ischemic times are more variable, with patients often presenting later. In such cases it is difficult to be certain whether the findings from our prospective cohort would still apply.

**Conclusions.** Our results confirm the prognostic significance of lymphopenia following PPCI for STEMI and demonstrate a characteristic pattern of lymphocyte subset depletion following reperfusion. Crucially, our data also suggest a role for effector T cells in the development of MVO and myocardial I/R injury. We have identified the chemokine fractalkine as a prime candidate to have a critical function in this process. The clinical implications of these findings are potentially far-reaching. No effective treatment is currently in use to ameliorate I/R injury in patients following PPCI. Our findings identify a potential therapeutic target, opening up a new avenue for further research and future treatment development.

**Methods**

**Supplemental Methods.** For further details, see Supplemental Methods.

**Retrospective clinical data collection and analysis (cohort 1)**

All STEMI patients meeting standard diagnostic criteria and undergoing PPCI (n = 1,531) admitted to a large tertiary center in the United Kingdom between April 2008 and February 2010 were identified retrospectively. Patients with in-hospital mortality were excluded, as were those with acute inflammatory or infectious disease, organ transplantation, or unavailable data, which left a total of 1,377 patients discharged alive and included in the final analysis.

The blood results (complete blood counts) of each patient were identified, and those closest to the time of PPCI and after 24 and 48 hours were recorded, as well as the minimum lymphocyte count during the admission. Mortality data were recorded up to July 2011, giving a follow-up of 40 months (mean 25.2 months).

Survival distributions were estimated using the Kaplan-Meier method and compared using the log-rank test. The effects of known prognostic variables on long-term mortality were examined using Cox proportional hazards regression analysis, using the covariates of sex, age, and minimum lymphocyte tertile, as well as all other baseline variables that differed significantly between groups (for the full list, see Supplemental Methods).

**Prospective lymphocyte subset characterization data (cohort 2)**

**Patient populations and blood sampling.** A cohort of 59 STEMI patients were prospectively identified and enrolled in the study at the time of admission. Inclusion criteria were chest pain of onset within 6 hours with new ST segment elevation. Exclusion criteria included cardiogenic shock, previous MI, active infection or malignancy, chronic inflammatory conditions, patent arterial flow in the infract related artery, and any contraindication to cardiac MRI scanning (for the full list, see Supplemental Methods).

Coronary angiography and PPCI were performed, and arterial blood was acquired at the start of the procedure, then at 15, 30, and 90 minutes following reperfusion. Venous blood was obtained at 24 hours, as well as at 3 to 6 months in a subset of 23 patients.

In a subset of 12 STEMI patients, upon completion of the PPCI procedure and as close as 30 minutes following reperfusion as possible, blood was obtained from the CS via catheterization of the femoral or brachiocephalic vein. Catheter position within the CS was confirmed radiographically by contrast injection. "Simultaneous" aortic blood was obtained via the angioplasty catheter within 30 seconds of CS sampling.
A control group of 15 patients admitted with NSTEMI undergoing non-emergency angiography with or without percutaneous coronary intervention (PCI) were also enrolled. Arterial blood was acquired from these patients at the start of the procedure and at 15, 30, and 90 minutes after culprit vessel instrumentation (or after initial sampling in 4 cases without intervention). A further 5 NSTEMI patients undergoing PCI were subsequently recruited for analysis of lymphocyte chemokine receptor expression.

Enumeration of major leukocyte populations. Blood was obtained as above in 4 ml EDTA tubes (BD Biosciences). Absolute counts of granulocytes, monocytes, total lymphocytes, CD3+ lymphocytes, and T lymphocytes, as well as the major subsets CD4+ and CD8+ T cells, were determined using a four-color BD TruCount flow cytometric assay (BD Biosciences, #342447), as described previously (Supplemental Figure 4, Supplemental Table 4 for CV values for this assay, and ref. 34).

Enumeration of detailed leukocyte subpopulations. Absolute counts of detailed leukocyte subpopulations were determined using an eight-color flow cytometric assay, in conjunction with the major population counts obtained as above. Briefly, 50-μl aliquots of whole blood were stained with a cocktail of the following antibodies: CD3-FITC (clone UCH-T1, #555332), CD4-V500 (clone RPA-T4, #560768), CD8-APC-H7 (clone SK1, #641400), CD16-PE (clone B73.1, #561313), CD27-APC (clone L128, #337169), and CCR7-PE-Cy7 (clone 3D12, #557648) (all BD Biosciences); CD45RA-Pacific Blue (clone MEM-56, #MHCDF45RA28, Invitrogen); and CD56-PerCP-eFluor710 (clone CMSSB, #42-0567-42, eBioscience). The samples were then lysed using Pharm Lyse (BD Biosciences) according to the manufacturer’s instructions, followed by three wash steps using a BD Lyse Wash Assistant machine (BD Biosciences). Analysis was performed using a BD FACSCanto II machine with FACSDiva software (BD Biosciences). T cell subclassification was carried out on the established model proposed by Sallusto et al., dividing both CD4+ and CD8+ T cells into naive (Tn), central memory (Tcm), effector memory (Tem), and effector memory (Temra) cells (Figure 8 and refs. 15, 35, 36). The cell count for each subset was calculated as the count of the parent population (derived from TruCount assay above) multiplied by the percentage of the parent cells within the subset gate. For CV values for this assay, see Supplemental Table 11.

Serum fractalkine quantification (ELISA). Blood samples were obtained in Vacutainer Serum Tubes (BD Biosciences) and allowed to clot at room temperature. Within 4 hours of collection, serum was separated by centrifugation at 1,000 g at room temperature for 10 minutes and stored at −80°C until further analysis. Soluble fractalkine was measured in serum samples with the ELISA kit (Human CX3CL1/Fractalkine immunoassay, #DCX310, R&D Systems), according to the manufacturer’s instructions.

T cell chemokine receptor expression. The surface expression of chemokine receptors in T cells was assessed using a six-color flow cytometric assay. Aliquots of 100 μl whole blood were incubated with a cocktail of the following four antibodies: CD3-PE (clone UCH-T1, #555333), CD4-V400 (clone RPA-T4, #560768), CD8-FITC (clone RPA-T8, #555366) (all BD Biosciences), and CCR7-BV421 (clone G043H7, #355208, BioLegend), as well as an APC-labeled antibody of one of the following specificities: CCR2 (clone K036C2, #357212), CCR4 (clone L291H4, #359420), CCR5 (clone J418F1, #359108), CCR6 (clone G034E3, #353418), CXCR4 (clone 12G5, #306518), CXCR5 (clone J252D4, #356924), or CXCR6 (clone K041E5, #356012) (all BioLegend). Lysis was performed using Pharm Lyse, followed by 3 wash steps as described above. Analysis was then performed on a BD FACSCanto II cytometer, and expression of the each chemokine receptor determined using mean fluorescence intensity (MFI) on the appropriate channel. CD4+ and CD8+ T cells in this assay were further sub-divided into CCR7+ (Tn and Tcm combined) and CCR7− (Tem and Temra) cells.

Leukocyte surface CX3CR1 expression in STEMI time-course assay. The time-course of expression of CX3CR1 on leukocyte subsets in STEMI was determined using a six-color flow cytometric assay. Aliquots of 100 μl whole blood were stained with a cocktail of the following antibodies: CD3-FITC (clone UCH-T1, #555352), CD4-V500 (clone RPA-T4, #560768), CD8-APC-H7 (clone SK1, #641400), CD16-PE (clone B73.1, #561313), CD27-APC (clone L128, #337169), and CCR7-PE-Cy7 (clone 3D12, #557648) (all BD Biosciences); CD45RA-Pacific Blue (clone MEM-56, #MHCDF45RA28, Invitrogen); and CD56-PerCP-eFluor710 (clone CMSSB, #42-0567-42, eBioscience). The samples were then lysed using Pharm Lyse (BD Biosciences) according to the manufacturer’s instructions, followed by three wash steps using a BD Lyse Wash Assistant machine (BD Biosciences). Analysis was performed using a BD FACSCanto II machine with FACSDiva software (BD Biosciences). T cell subclassification was carried out on the established model proposed by Sallusto et al., dividing both CD4+ and CD8+ T cells into naive (Tn), central memory (Tcm), effector memory (Tem), and effector memory (Temra) cells (Figure 8 and refs. 15, 35, 36). The cell count for each subset was calculated as the count of the parent population (derived from TruCount assay above) multiplied by the percentage of the parent cells within the subset gate. For CV values for this assay, see Supplemental Table 11.

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Cardiac MRI. MRI scans were obtained at 1–8 days after infarction with a Siemens Avanto 1.5 Tesla MRI scanner, using a phased array body coil. All images were obtained during breath-holding. Cine images were obtained in parallel short axis slices covering the full extent of the left ventricle, as well as vertical and horizontal long axis, and three chamber views, using a steady-state free precession pulse (SSFP) sequence (repetition time [TR]: set according to heart rate, echo time [TE]: 1.19 ms, flip angle: 80°, field of view [FOV]: 255 ×
340 mm, image matrix: 144 × 192). Corresponding end-diastolic short axis LGE images were obtained using an inversion recovery (IR) segmented gradient echo sequence (TR: according to heart rate, TE: 3.41 ms, flip angle: 25°, FOV: 255 × 340 mm, matrix: 192 × 256) 10 minutes after administration of gadobutrol contrast (Gadovist, Bayer Schering Pharma) at a dose of 0.1 mmol/kg. The inversion time (TI) was selected and adjusted throughout to null normal myocardium.

Image analysis was performed using validated cardiac MRI analysis software (cvii+, Circle Cardiovascular Imaging Inc.) by an individual blinded to cellular data. Following visual identification of the limits of the left ventricular (LV) cavity, epicardial and endocardial borders were traced automatically on each end-systolic and end-diastolic short axis cine frame, with manual correction where necessary, allowing calculation of LV mass, dimensions, and ejection fraction (LVEF). Epicardial and endocardial borders were then traced on each LGE slice, and areas of enhancement (infarction) identified and quantified automatically using a signal intensity threshold of 5 SDs above normal remote myocardium, as previously described and validated (37). Regions of hypoenhancement within the enhanced zone (MVO) were identified and quantified using semiautomatic thresholding following manual border delineation of areas of interest, and included in the calculated infarct mass.

Statistics

All statistical analysis was performed using SPSS (version 21; IBM), and graphs were produced in GraphPad Prism (version 6). Where data did not pass normality testing by Shapiro-Wilk test, nonparametric correlations between parameters were assessed using Spearman correlation coefficient. Unmatched groups were compared using Mann-Whitney U test (two groups) or Kruskal-Wallis test with Dunn’s multiple-comparisons test (three or more groups). Matched groups (three or more) were compared using Friedman’s test with Dunn’s multiple-comparisons test. Where normality testing criteria were met, three or more groups were compared using one-way ANOVA with Holm-Šidák multiple-comparisons test. Data are expressed as mean ± SEM unless otherwise stated. Where multiple comparison tests were used, reported P values are those corrected for multiple tests. A P value of less than 0.05 was considered significant.

Study approval

For the prospective component, ethical approval was obtained from the National Research Ethics Service Committee North East (REC ref. 12/NE/0322). The study was conducted according to the principles set out in the Declaration of Helsinki. Written informed consent was obtained from all prospective patients.

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