Monocytes in Ischemic heart disease
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Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir


Submitted

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ABSTRACT

**Aims** Monocytes are critical mediators of healing following AMI, making them an interesting target to improve myocardial repair. The purpose of this study was gain insight into the source and recruitment of monocytes following acute myocardial infarction (AMI) in humans.

**Methods and results** Post-mortem tissue specimens of myocardium, spleen and bone marrow were collected from 28 patients that died at different time points after AMI. Twelve patients that died from other causes served as controls. Presence and localization of monocytes (CD14\(^+\) cells), and their CD14\(^+\)CD16\(^-\) and CD14\(^+\)CD16\(^+\) subsets were evaluated by immunohistochemical and immunofluorescence analyses. CD14\(^+\) cells localized at distinct regions of the infarcted myocardium at different time points following AMI. At 12 h–5 days after AMI, CD14\(^+\) cells were predominantly located in the infarct border zone, adjacent to cardiomyocytes, and consisted for 85% [78–92] of CD14\(^+\)CD16\(^-\) cells. In contrast, 5–14 days after AMI, massive accumulation of CD14\(^+\) cells was observed in the infarct core, containing comparable proportions of the CD14\(^+\)CD16\(^-\) subset (60% [31-67]) and the CD14\(^+\)CD16\(^+\) subset (40% [33-69]). Importantly, in patients with AMI, of the number of CD14\(^+\) cells was decreased by 39% in the bone marrow and by 58% in the spleen, in comparison to control patients \((P=0.02\) and \(P<0.001\), respectively).

**Conclusions** Overall, this study showed a unique spatio-temporal pattern of monocyte accumulation in the human myocardium following AMI that coincides with a marked depletion of monocytes from the spleen, suggesting that the human spleen contains an important reservoir function for monocytes.
INTRODUCTION

In patients with acute myocardial infarction (AMI), an adequate healing response is crucial for preserving left ventricular (LV) function and geometry, and thus preventing adverse LV remodeling.\(^{1,2}\) Infarct healing is a complex and dynamic process, consisting of replacement of necrotic myocardium with scar tissue, and is critically mediated by monocytes.\(^{3-5}\) Accordingly, monocytes have recently drawn considerable attention as a target to improve post-AMI repair.\(^{5,7}\)

Human peripheral blood monocytes are a heterogeneous pool of cells, consisting of at least two subsets, the CD14\(^+\)CD16\(^-\) and CD14\(^+\)CD16\(^+\) monocytes, which have unique characteristics with regard to phenotype and function.\(^8\) Although circulating monocytes, once infiltrated into the infarcted myocardium, are generally referred to as macrophages, results from mice studies indicate that monocytes may have distinct fates.\(^9\) That is, infiltrated monocytes, while pursuing their functions, may eventually differentiate into macrophages, emigrate from the site of injury, or die by apoptosis or necrosis, and are rapidly replenished by newly recruited monocytes. It has been recently shown after coronary ligation in mice, that a large proportion of these newly recruited monocytes is provided by the spleen, suggesting a unique extramedullary reservoir function for monocytes.\(^{10}\)

While monocytes critically mediate infarct healing, there is a growing body of evidence indicating that uncontrolled monocyte response may impair post-AMI healing and directly affect prognosis.\(^{11,12}\) In AMI patients, blood levels of CD14\(^+\)CD16\(^-\) monocytes peak at day 3, whereas levels of CD14\(^+\)CD16\(^+\) monocytes peak on day 5.\(^{13}\) Several clinical studies reported that patients with high blood levels of CD14\(^+\)CD16\(^+\) monocytes following AMI show poor functional outcome,\(^{7,13}\) suggesting that excessive recruitment of particularly CD14\(^+\)CD16\(^+\) monocytes may enhance post-AMI injury. However, it remains unclear whether the dynamic changes of monocyte subsets levels, observed in blood, reflect the cell’s presence in the infarcted tissue.

So far, animal studies have provided important insights into the origin, source, recruitment and functions of monocytes in post-AMI healing,\(^{9,10,14,15}\) and clinical studies even provided a rationale for future therapeutic strategies.\(^{7,13}\) However, histological data of patients are lacking. To address these issues, we performed detailed histological analyses of clinical autopsy material to gain insights into the systemic monocyte response following AMI in patients.

METHODS

Patients, tissue collection and processing
A total of 40 patients that were referred to the department of Pathology, VU University Medical Centre (VUmc; Amsterdam, the Netherlands) for clinical autopsy were included in this study. Clinical autopsy was performed within 24 hours after death. Twenty eight patients were diagnosed at clinical autopsy with recent left ventricle AMI. All these patients showed macroscopical evidence for recent left ventricle AMI, as identified by lactate dehydrogenase (LDH) decolouration of the injured
myocardial tissue (Figure 1A). Macroscopic heart slides were photographed and the surface of the LDH decolourized area in relation to the surface of the left ventricle and septum was used to determine the percentage of infarct size. Furthermore, based on LDH decolouration, the infarct area was classified as subendocardial or transmural infarction. Twelve patients, who died from a cause not related to AMI, and thus, with macroscopic normal LDH staining, served as controls. All patients but one with evidence for sepsis, myocarditis, metastasized cancer, recent cerebrovascular accident or recent pulmonary embolism were excluded, as these conditions are known to influence the number of monocytes in the myocardium, spleen or bone marrow.9,16-19 One patient died immediately after large pulmonary embolism in the proximal part of the pulmonary artery, indicative for sudden death, and was therefore included in the control group. Furthermore, lung tissue was examined for histopathological evidence of pneumonia at time of death. The present study was conducted in accordance with the Declaration of Helsinki. The study protocol (CASIMIR) was approved by Research Committee of the Department of Pathology of the VUmc. Use of autopsy material after completion of the diagnostic process is part of the patient contract in the VUmc.

Myocardial tissue specimens were obtained from the centre of the infarct area (left ventricle) in AMI patients, as identified by LDH decolouration (Figure 1A). In control patients, myocardial tissue specimens were obtained from the left ventricle. From each patient, also a tissue sample from a thoracic vertebral body (bone marrow) and from the subcapsular part of the spleen was collected, as this area was previously reported to be the site of a monocytic reservoir in mice.10 Tissue specimens were formalin-fixed and paraffin-embedded for immunohistochemical analyses. Myocardial tissue specimens of 19 AMI patients were also snap frozen, and stored at -196˚C (liquid N2) for immunofluorescence analyses.

In the AMI patients, the time between onset of AMI and death was estimated using microscopic criteria, and categorized into three phases of post-AMI healing: 3–12 h after AMI (macroscopically LDH decolouration but no extravasation of neutrophilic granulocytes in the infarct area; n=9), 12 h–5 days after AMI (extravasation of neutrophilic granulocytes in the infarct area; n=9) and 5–14 days after AMI (granulation tissue formation; n=10), which corresponds to the so-called post-AMI early, inflammatory and proliferative phase, respectively.20-22

To identify multivessel disease, haematoxylin and eosin (HE) stainings of the three coronary arteries (left anterior descending (LAD) artery, left circumflex (LCX) artery, and right coronary artery (RCA)) were used to microscopically determine the rate of stenosis in the artery. Patients that contained two or three coronary arteries with more then 50% stenosis were classified as containing multivessel disease.

**Immunohistochemistry**
Deparaffinised and rehydrated sections of myocardium, spleen and bone marrow were incubated in methanol/H₂O₂ (0.3%) for 30 minutes to block endogenous peroxidases. Antigen retrieval was performed by heating in Tris-EDTA buffer (pH 9.0). Sections were then incubated with anti-human CD14 (1:40; clone 7, Novocastra, Newcastle Upon Tyne, United Kingdom). The immunostaining was revealed by using the EnVision Detection kit (Dako, Copenhagen, Denmark). Staining was
visualized using 3,3'-diaminobenzidine (DAB, 0.1 mg/ml, 0.02% H$_2$O$_2$), and sections were counterstained with haematoxylin, dehydrated and covered. For the negative controls the primary antibody was replaced by phosphate-buffered saline. These sections were all found to be negative.

Monocytes were identified as CD14$^+$ cells. Endothelial cells and neutrophils were found to stain negative for CD14. Stained myocardial tissue sections were scanned with a Mirax slide scanner system using a 20× objective (3DHISTECH, Budapest, Hungary). Numbers of CD14$^+$ cells were determined and equated for areas. Notably, in the infarct area of 12 h–5 days old and the infarct area of 5–14 days old two areas can be identified. We defined the microscopical infarct core as the area consisting of necrotic tissue with infiltrating neutrophilic granulocytes in AMI of 12 h–5 days old, and of granulation tissue in AMI of 5–14 days old. The microscopical border zone was defined as the area adjacent to the microscopical infarct core, containing the viable cardiomyocytes (Figure 1B). In stained spleen tissue sections, numbers of CD14$^+$ cells were quantified per surface area, which was measured using Q-PRODIT (Leica, Cambridge, UK). In stained bone marrow sections, numbers of CD14$^+$ cells were determined in a minimum of 10 high-power-fields (400x magnifications).

**Immunofluorescence**
Sections of frozen myocardial tissues were fixed in 3% paraformaldehyde (Sigma-Aldrich Co., St. Louis, MO, USA) followed by incubation with 10% normal goat serum (Dako, Copenhagen, Denmark). Slides were incubated with the primary antibodies at 4°C: mouse anti-human CD14 (1:50; clone: M5E2 (IgG$_2a$); BD Pharmingen, San Diego, CA, USA), mouse anti-human CD16 (1:20; clone: 3G8 (IgG$_1$); Molecular Probes, Leiden, The Netherlands); mouse anti-human α-actinin (1:100; clone: EA-53 (IgG1), Sigma Aldrich, St. Louis, MO, USA); rabbit anti-human C3d (1:1000, Dako). Subsequently, slides were incubated with the appropriate secondary antibodies: goat anti-mouse IgG$_{2a}$ Alexa Fluor 488; goat anti-mouse IgG, Alexa Fluor 647; goat anti-mouse IgG1 Alexa Fluor 568; goat anti-rabbit IgG (H+L) Alexa Fluor 568 (all 1:100, Molecular Probes) and counterstained with Hoechst 33342 (1:1000, Molecular Probes).

Stained myocardial sections were examined under the Leica DM6000 microscope (Leica Microsystems, Heidelberg, Germany). Monocytes were identified as CD14$^+$ cells. Two subsets were distinguished by the expression of CD16. The proportion of CD14$^+$/CD16$^-$ cells and CD14$^+$/CD16$^+$ cells per area was determined in 5-10 microscopic fields (200x magnifications). These proportions combined with the absolute number of CD14$^+$ cells, as measured by immunohistochemical analyses, were used to estimate the numbers of CD14$^+$/CD16$^-$ cells and CD14$^+$/CD16$^+$ cells in distinct areas.

**Statistical analysis**
Statistical analysis was performed with Statistical Package for Social Sciences software (SPSS 16.0 for Windows, SPSS Inc). The Fisher’s exact test and the Freeman-Halton extension of Fisher’s exact test were used for testing associations between categorical data. To test for differences between groups, the Kruskal-
Wallis test or Mann-Whitney U test were used for continuous data, unless indicated otherwise. Linear nonparametric correlation was calculated using the Spearman correlation. Results were considered statistically significant if the two-sided $P$-value was <0.05.

**RESULTS**

**Patients**

Samples of myocardium, spleen and bone marrow taken from 28 patients that died after AMI were studied. The characteristics of these patients are shown in Table 1. Mean age was 66 ± 13 years, 75% was male. Thirteen patients had a previous AMI, 6 patients were diagnosed with diabetes mellitus type II, 2 patients with chronic kidney disease, and 11 patients had multivessel disease.

**Unique spatio-temporal pattern of CD14+ cell accumulation following AMI**

To investigate the sequential accumulation of monocytes following AMI and their regional distribution, we first performed detailed immunohistochemical analyses of CD14+ cells in the infarct area of patients that died at different time points after AMI (Figure 2A and 2C). At 3–12 h after AMI, the presence of CD14+ cells in the infarct area was comparable to control myocardium (infarct area: 3.5 cells/mm² [1.9–5.4]; control: 5.0 cells/mm² [3.2–8.9]; $P=0.11$), indicating an absence of additional influx of CD14+ cells early after AMI. Thereafter, at 12 h–5 days after AMI, CD14+ cells predominantly accumulated in the infarct border zone, adjacent and also adherent to cardiomyocytes (Figure 2B), and to a much lesser extent in the necrotic infarct core (border zone: 63.9 cells/mm² [33.6–90.2]; infarct core: 13.9 cells/mm² [4.8–20.5]; $P=0.007$). In contrast, at 5–14 days after AMI, large numbers of CD14+ cells were

![](image1.jpg)

**Figure 1** Identification of the infarct area by LDH decolouration, and the microscopical infarct core and border zone within the infarcted myocardial tissue. (A) Photograph of LDH coloured myocardium. The dotted line outlines the LDH decoloured infarct area. The square indicates the centre of the infarct area (including the microscopical infarct core and border zone), wherefrom tissue was sampled. (B) HE staining of the infarcted myocardial tissue (5–14 days old) was performed to identify two areas within the infarct area: the microscopical infarct core and border zone (50x magnifications). BZ denotes border zone; IC, infarct core.
almost exclusively present in the infarct core, consisting of granulation tissue at this point of time after AMI (infarct core: 149.4 cells/mm$^2$ [103.1–501.8]; border zone: 20.4 cells/mm$^2$ [12.0–50.4]; $P<0.001$). These data reveal a distinct spatio-temporal pattern of monocyte accumulation following AMI.

**Accumulation of CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ cells following AMI**

Monocyte subsets have been attributed diverse functions in the post-AMI healing process. Therefore, we subsequently analyzed the proportions of CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ cells in the CD14$^+$ cell infiltrate in the border zone at 12 h–5 days after AMI, and in the infarct core at 5–14 days after AMI (Figure 3). At 12 h–5 days after AMI, 85% [78–92] of the CD14$^+$ cells in the infarct border zone were CD14$^+$CD16$^-$

### Table 1 Patient characteristics.

<table>
<thead>
<tr>
<th>Control (n=12)</th>
<th>AMI (n=28)</th>
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<tr>
<td></td>
<td>3–12 h</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Male gender</td>
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<tr>
<td>Medical history</td>
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<tr>
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<tr>
<td>Cerebral vascular accident</td>
<td>1 (8)</td>
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<tr>
<td>Chronic kidney disease</td>
<td>0 (0)</td>
</tr>
<tr>
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<td>2 (17)</td>
</tr>
<tr>
<td>Cancer</td>
<td>1 (8)</td>
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<tr>
<td>Primary reason of death</td>
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<tr>
<td>Acute myocardial infarction</td>
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</tr>
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</tr>
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<tr>
<td>Vitil bleeding</td>
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<tr>
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<tr>
<td>Arrhythmia (not associated with AMI)</td>
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<tr>
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</tr>
<tr>
<td>Acute large pulmonary embolism</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>1 (8)</td>
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<tr>
<td>Trauma</td>
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<td>In-hospital death</td>
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<td>Successful reperfusion therapy</td>
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<tr>
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<tr>
<td>Extent of infarction (%)</td>
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<td>Pneumonia</td>
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</tbody>
</table>

Data are presented as mean ± SD or number (%) of patients. AMI denotes acute myocardial infarction; h, hours; vs, versus. One-way ANOVA $P$-value; $^*$Student’s t-test $P$-value; $^*$Analysis included 5 patients; $^*$Analysis included 6 patients; $^*$Analysis included 7 patients; $^*$Analysis included 8 patients.
cells. In contrast, at 5–14 days after AMI, comparable proportions of CD14⁺CD16⁻ cells (60% [31-67]) and CD14⁺CD16⁺ cells (40% [33-69]) were observed in the infarct core (Figure 4A). In Figure 4B, the absolute numbers of CD14⁺CD16⁻ and CD14⁺CD16⁺ cells are depicted.

**CD14⁺ cells in the spleen and bone marrow following AMI**

Although monocytes originate in the bone marrow, recent experimental studies indicate that the majority of monocytes are recruited from the spleen following AMI in mice.¹⁰ Because of this, we also investigated the numbers of CD14⁺ cells in the bone marrow and the spleen. In the total group of AMI patients, we observed a 39%

![Figure 2](image_url)
Systemic monocyte response following AMI

decrease of CD14+ cells in the bone marrow and a 58% decrease of CD14+ cells in the spleen, as compared to the control group (P=0.02 and P<0.001, respectively). Figure 5A and 5B show the numbers of CD14+ cells in the bone marrow and the spleen, stratified according to the three different time points after AMI. Only in the spleen, the number of CD14+ cells was significantly lower at all time points after AMI, when compared to the control group, even early after AMI (3–12 h). Of note, no significant association was found between the extent of infarction and the number of CD14+ cells in the spleen (Spearman’s r = 0.09, P=0.69) and the bone marrow (Spearman’s r=0.02, P=0.92).

DISCUSSION

Recent studies point to an important role of monocytes in post-AMI healing and adverse LV remodelling, making them an interesting target to improve myocardial repair.6,7 To gain more insight into the source and recruitment of monocytes following AMI in patients, we conducted an autopsy study and found that CD14+ cells accumulate in distinct regions of the infarcted myocardium at different time points following AMI. We showed that CD14+ cells, recruited 12 h–5 days after AMI, predominantly accumulate in the infarct border zone and are located adjacent and also adherent to cardiomyocytes. In contrast, 5–14 days after AMI, CD14+ cells almost exclusively invade the infarct core, consisting of granulation tissue. Analysis of CD14+ cell subsets showed abundant presence of the CD14+CD16– subset in the border zone at 12 h–5 days after AMI, whereas comparable proportions of CD14+CD16– and CD14+CD16+ cells were present in the infarct core at 5–14 days. A decrease of CD14+ cells was observed in bone marrow, but especially in the spleen following AMI, suggesting for the first time that the spleen may constitute an extramedullary reservoir of monocytes in humans. Taken together, these observations clarify the post-AMI monocyte response in patients and may provide new clues for treatment.

In the past decades, substantial progress has been made in understanding the complex roles of monocytes in healing after AMI. Tsujioka et al. measured the levels of CD14+CD16– cells and CD14+CD16+ cells in the blood of AMI patients, and found that the CD14+CD16– subset peaks at day 3 after AMI, whereas the CD14+CD16+ subset peaks at day 5. The same group reported that post-AMI myocardial salvage was decreased in patients with high peak levels of circulating CD14+CD16– cells.13 It has been suggested that excessive accumulation of CD14+CD16+ cells following AMI may enhance myocardial inflammation, leading to infarct expansion and adverse LV remodeling.6

Our data now reveal that CD14+CD16+ cells are not only increased in the blood but also in the infarct area. More importantly, at 12 h–5 days after AMI, we show that CD14+CD16+ cells primarily accumulate in the border zone adjacent but also adhering to cardiomyocytes, surrounding the necrotic infarct core. Phagocytosis of dead cells and debris is considered the major function of CD14+CD16+ cells during the post-AMI healing process. Therefore, our finding raises several questions: (1) why do CD14+CD16+ cells primarily invade the infarct border zone (and not the necrotic infarct core), and (2) what is their biological role in this area? Particularly
cardiomyocytes in the border zone are at risk for post-AMI apoptosis, due to inflammation. In this regard, it is intriguing to speculate that CD14\(^+\)CD16\(^-\) cells may contribute to the fate of the cardiomyocytes that survived the primary ischemic period. As protection of viable cardiomyocytes following AMI is considered a “holy grail” in cardiovascular medicine, future studies that unravel the role of CD14\(^+\)CD16\(^-\) cells in the border zone are warranted.

The relevance of the CD14\(^+\)CD16\(^+\) subset in infarct healing is less clear. CD14\(^+\)CD16\(^+\) cells consist of multiple subpopulations with different characteristics. No significant associations have been found between the blood level of CD14\(^+\)CD16\(^+\) cells in AMI patients and parameters of functional outcome. Our results show abundant presence of CD14\(^+\)CD16\(^+\) cells in the infarct core at 5–14 days after AMI,
at the same time as the previously reported increase of CD14^+CD16^+ cells in the blood. Their location within the infarct core, more specific, in granulation tissue, support a role for CD14^+CD16^+ cells in angiogenesis and subsequent scar formation. Of relevance, the proportion of CD14^+CD16^+ cells in the infarct core at this time after AMI of 40% [33–69] is much higher than the proportion of circulating CD14^+CD16^+ cells in the blood of approximately 10–15% reported in the clinical studies, suggesting that CD14^+CD16^+ cells are selectively recruited, which could explain the lack of association between the level of CD14^+CD16^+ cells in blood and parameters of functional outcome in patients.

Monocytes were thought to originate in the bone marrow from hematopoietic stem cells and progenitor cells, and subsequently enter the circulation where they are made available to sites of tissue injury. In support of this, we observed reduced numbers of CD14^+ cells in the bone marrow of AMI patients, indicating release of monocytes in response to cardiac injury. Importantly, recent studies also point to the spleen as a major source of monocytes. In 2009, Swirski et al. showed that the murine spleen stores large amounts of monocytes, which can be recruited to sites of injury. In accordance with these studies in mice with coronary ligation, numbers of CD14^+ cells in the spleen of patients were profoundly decreased at all time points following AMI. Even early after AMI (3–12 h) numbers of splenic CD14^+ cells were already diminished, suggesting that the human spleen contains an important reservoir function for monocytes that can be rapidly deployed. This could also provide a possible explanation of why levels of monocytes in blood are already increased at hospital admission of patients with AMI or ischemic stroke.
Interestingly, Swirski et al. also demonstrated in their preclinical studies that the release of splenic monocytes into the circulation is mediated by angiotensin II, and not the chemokine (C-C motif) receptor 2, which mediates mobilization of monocytes from bone marrow\textsuperscript{10}, suggesting that the mobilization of monocytes from the spleen depends on different cues compared to those from the bone marrow. Furthermore, recent studies have shown that the spleen not only stores and releases monocytes, but also supports post-AMI extramedullary monocyte production, further underscoring the role of the spleen in the monocyte response following AMI\textsuperscript{9,29} Hence, increased understanding of the mechanisms that regulate storage, production and release of splenic monocytes in AMI patients may provide new perspectives for the development of therapeutic strategies that target the monocyte response and infarct healing.

**Study limitations**
There are several limitations to the present study. First, the results were solely observational in nature, and future clinical studies are necessary to confirm our findings, using advanced cell labelling and imaging techniques. Second, our sample size might be too small to detect small differences between groups. Third, it cannot be excluded that findings of this autopsy study might be different in survivors of AMI, due to conditions/complications associated with death that may have influenced the systemic monocyte response. Fourth, detailed premortem clinical data was lacking. Fifth, frozen tissue was only collected from the myocardium of 19 AMI patients, and not from the spleen and bone marrow. Notably, it was not possible to cut frozen bone marrow, because the bone needed to be decalcified. Therefore, CD14\textsuperscript{+} cell subset analysis was performed only in the myocardium of AMI patients. Sixth, in this study it was not possible to distinguish monocytes from macrophages.

**Conclusions**
Overall, this study uncovers several novel aspects regarding the monocyte response following AMI in patients, such as the influx of CD14\textsuperscript{+}CD16\textsuperscript{−} cells in the border zone at 12 h–5 days after AMI, surrounding the necrotic infarct core, and the presence of CD14\textsuperscript{+}CD16\textsuperscript{+} cells in the granulation tissue (infarct core) at 5–14 days. Furthermore, this study for the first time suggests that monocyte recruitment to the site of infarction coincides with depletion of monocytes from the spleen, indicating that the human spleen contains an important reservoir function for monocytes, as previously shown in mice with AMI\textsuperscript{10}. Future studies, are needed to increase knowledge on the dynamics of monocyte subsets in the myocardium using advanced cell labelling and imaging techniques, and to increase understanding of their biological roles, and the mechanisms that control monocyte recruitment following AMI in patients.

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